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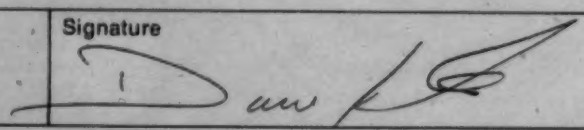

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Development of Photochemical Activity in Relation to Pigment and Membrane Protein Accumulation in Chloroplasts of Greening Gateway Barley and its Virescens Mutant

by

© David J. Kyle

University of Alberta

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Doctor of Philosophy

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Development of Photochemical Activity in Relation to Pigment and Membrane Protein Accumulation in Chloroplasts of Greening Gateway Barley and its Virescens Mutant submitted by David J. Kyle in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Physiology and Biochemistry.

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Abstract

The development of photochemical activity in relation to pigment and membrane protein accumulation in chloroplasts of greening Gateway barley and its virescens mutant was studied. Seedlings of both genotypes were etiolated or grown under continuous illumination for 6 or 8 days. The chlorophyll a/b ratio was significantly lower in the mutant after both 6 and 8 days in continuous light. Upon exposure of etiolated seedlings to light, the rate of chlorophyll accumulation per plastid was faster in the normal than in the mutant seedlings after 6 days of etiolation. These rates were almost equal after 8 days due to a decrease in the rate of greening of the normal seedlings. A larger amount of extractable plastoquinone A per unit chlorophyll was present in the mutant at both 6 and 8 days. Although the protein content per plastid did not vary as a function of greening, the sodium dodecyl sulfate polyacrylamide gel electrphoresis showed a change in the polypeptide profiles. High molecular weight polypeptides (96000 and 66000) declined whereas those at 34000, 27000 and 22000 showed an increase during chloroplast development. Electrophoresis of thylakoid membranes treated with sodium dodecyl sulfate at 5C or 100C was used to identify chlorophyll-proteins. Chlorophyll-proteins corresponding to the reaction center of Photosystem I (mol wt 66000) and Photosystem II (mol wt 47000), as well as a monomer (mol wt 29000) and a dimer

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(55000) of the light-harvesting chlorophyll-protein complex were present in both normal and mutant thylakoids. Freeze-fracture electron microscopy revealed two exoplasmic face (EF) particle size classes in the normal chloroplast membranes, only the smaller of which was present in the mutant. In addition, protoplasmic face (PF) particles in the larger of two size classes were only present after 8 days of continuous illumination in the mutant, whereas both size classes were clearly visible in the normal thylakoids after only 6 days.

Photochemical activities associated with Photosystem I (tetramethyl-p-phenylenediamine-->methyl viologen) and with Photosystem I plus Photosystem II (diphenylcarbazide-->dichlorophenolindophenol) were measurable within the first hour of greening in both normal and mutant chloroplasts. This was followed by the appearance of the photoreduction of dichlorophenolindophenol with water as electron donor at the 2 hr stage and oxygen evolution (water-->ferricyanide) at the 6 hr stage of greening. In all cases, the developmental rates (per unit protein) were faster in the normal than in the mutant seedlings after 6 days of etiolation, but similar after 8 days due to a decrease in the developmental rate of the normal. There was no difference in the electron flow through the Photosystem II reaction centre (diphenylcarbazide-->dimethyl-p-benzoquinone) between normal and mutant seedlings grown in continuous light in spite of

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the higher levels of plastoquinone A in the mutant, and the higher rates of whole system photosynthetic electron transport (water-->ferricyanide) or water-->dichlorophenolindophenol) in the normal chloroplasts. The photosynthetic unit size measured by the kinetics of light saturation or dichlorophenyl-dimethylurea inhibition of diphenylcarbazide-->dichlorophenolindophenol, was the same in both and mutant seedlings. However, changes in size of the photosynthetic unit associated with development were slower in the mutant.

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Abbreviations

ALA	5-aminolevulinic acid
ATP	adenosine triphosphate
APS	ammonium persulfate
BSA	bovine serum albumin
Chl	chlorophyll
DAD	2,3,5,6,-tetramethyl-p-phenylendediamine
DBMIB	2,5,-dibromo-3-methyl-6-isopropyl-p-benzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DMQ	2,5-dimethyl-p-benzoquinone
DPC	diphenylcarbazide
DPIP	2,6-dichlorophenolindolphenol
DSPD	disalicylidenpropanediamine
EDTA	ethylenediamine tetraacetic acid
EF	exoplasmic face
EM	electron microscopy
EPR	electron paramagnetic resonance
FeCN	ferricyanide
HEPES	N¹-2-hydroxyethylpiperazine-N¹-2-ethanesulfonate
I50	50% inhibition
Kd	kilodaltons
LHC	light harvesting chlorophyll a/b complex
M	mutant (virescens) Gateway barley
MeOH	Methanol
MV	methyl viologen
N	normal (wild-type) Gateway barley
NADP	nicotinamide adenine dinucleotide phosphate
PAGE	polyacrylamide gel electrophoresis
PC	plastocyanin
PET	photosynthetic electron transport
PF	protoplasmic face
Pi	inorganic phosphate
PQ	plastoquinone
PSI	photosystem I
PSII	photosystem II
rRNA	ribosomal ribonucleic acid
RuBP	ribulose bisphosphate
SDS	sodium dodecyl sulfate
SEM	scanning electron microscope
SiMo	silicomolybdate
TEM	transmission electron microscope
TEMED	N,N,N¹,N¹-tetramethyl-ethylenediamine
TLC	thin layer chromatography
TMPD	N,N,N¹,N¹,-tetramethyl-p-phenylenediamine
TPIP	2,3¹,6-trichlorophenolindophenol
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid

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I. INTRODUCTION

An effective method of studying chloroplast morphogenesis involves the use of specific mutations which delay or halt development at a particular point in the sequence of events. Many such mutants have been characterized in barley by Von Wettstein (233). One such mutant, virescens, is phenotypically characterized by a seedling which is pale yellow-green upon emergence, but which later recovers and becomes a viable green plant.

A virescens mutant of Gateway barley has been studied extensively in this laboratory for several years. This mutant is characterized by a light and temperature affected lag in chlorophyll biosynthesis (149, 160). Low amounts of protochlorophyll observed in the mutant etiolated seedlings (149) are not due to an inadequate production of ALA or its precursors (197). Mutant etioplasts contain no prolamellar bodies and upon illumination are slow to develop an internal lamellar system (117). Photoreductive activity (105) and protein synthesis (195, 197) are affected in light grown mutant seedlings, but the rate of mobilization of reserves from the embryo is not (196). This variety of pleiotropic effects, however, results from a single-gene mutation in the nucleus (217, 237).

One of the purposes of this thesis was to further characterize the mutant with respect to photochemical activities, pigment and chloroplast membrane proteins. Many

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differences have already been shown between normal and mutant chloroplasts, however, most of these previous studies involved a direct comparison between light grown yellow (mutant) and green (normal) plants. As a result, the differences seen could be explained by differences in the developmental stage of the tissues being compared, rather than those due to the specific mutation. In order to circumvent this problem, much of the work presented in this thesis involves the use of etiolated normal and mutant seedlings and a study of the subsequent greening. The dark pretreatment was used in an attempt to achieve a developmental alignment, thus reducing the effects of developmental differences.

The second aspect of this study involved the use of the mutant as a tool with which to study the various phenomena of chloroplast development. When grown under continuous illumination, greening of the mutant occurs very slowly. Thus developmental changes occurring in the mutant may closely approximate the sequence of events in the normal proplastid to chloroplast transformation, but at a rate slow enough to make detection of sequential changes possible.

I have divided this thesis into three main sections. The studies of the development of membrane proteins, chlorophyll, and plastoquinone have been grouped together under 'components of photosynthetic electron transport'. The experiments on the development of various photochemical sequences of the photosynthetic electron transport chain

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have been grouped together under 'functional studies'. Finally, the analyses of chloroplast membranes by freeze-fracture electronmicroscopy, which were done in collaboration with Ms. E. Weretilnyk, are presented as an appendix to the thesis.

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II. LITERATURE REVIEW

A. Chloroplast Structure

1. Pigments

The most obvious structural component of any green tissue is the pigment responsible for this color, namely chlorophyll. The lipophilic chlorophyll molecule is exclusively located in the internal photosynthetic membranes which, in eukaryotes, are compartmentalized within the chloroplast. Two classes of chlorophyll, chlorophyll a and bacteriochlorophyll a, are the only two pigments known to be responsible for the actual energy conversion step in photosynthesis in chlorophyll-containing organisms. The universality of chlorophyll can probably be explained by its unusual, but ideal, chemical structure. Chlorophyll is made up of four substituted pyrrole rings arranged in a macrocyclic structure surrounding a chelated magnesium ion. To this porphyrin core are attached various side chains, including the C20 isoprenoid alcohol, phytol. The pi-electron system of the molecule allows a strong absorption of visible light, while the aromatic stability of the ring structure provides a molecule which can "store" the energy of an absorbed photon and become a powerful electron donor. Although the chlorophyll molecule is complex in nature, it is synthesized from glycine and succinate (250) or glutamate (21, 158). These two compounds combine in a reaction catalyzed by ALA synthetase to produce

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aminolevulinic acid, the building block of chlorophyll. Indeed, the macrocycle of chlorophyll is simply an octamer of ALA. The biosynthetic pathway of chlorophyll has been well characterized for many species (see reviews 20, 28, 85, 188, 193)

Chlorophyll b in most higher plants, and phycobilins of blue green algae, are additional pigments which are secondarily involved in photosynthesis. These "antennae" molecules serve to trap light energy and transfer it to chlorophyll a and ultimately to the reaction centre (122). The carotenoids, a more diverse group of compounds, are found in most green species and are thought to act either as antennae or in the protection of chlorophyll from photooxidation (6, 80, 191). Chlorophylls and carotenoids also express the ability to bind to proteins (222) and therefore likely exist in the photosynthetic membrane in the form of pigment/protein complexes.

All of the enzymes involved in pigment biosynthesis have been found within the chloroplast (28, 85). However, inhibitor studies have shown that many of these enzymes are transcribed on cytoplasmic 80 S ribosomes in barley (127). The biosynthesis of chloroplast pigments, therefore, involves a dynamic interplay between the chloroplast and nuclear genomes.

2. Proteins

Protein makes up from 60-70% of the dry weight of the chloroplast (133). About half of this chloroplast protein is

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the soluble enzyme Ribulose-1,5-bisphosphate carboxylase. Most of the remaining protein is associated with the prolific membrane system of the plastid and can only be solubilized with detergents. One dimensional electrophoretic separations of thylakoid membranes in SDS have generated polypeptide profiles with as many as 43 distinct bands (106). Recently, two dimensional techniques have been developed involving isoelectric focusing in the first dimension followed by SDS-electrophoresis in the second (173). This method was first used on photosynthetic membranes to characterize subchloroplast fractions enriched in either PSI or PSII (172). Later, it was used to analyze the total complement of thylakoid proteins, with the result that 58 distinct peptide spots were identified from Chlamydomonas thylakoid membranes (30).

The identification of the membrane proteins seen on electrophoretic gels is difficult, as the removal of proteins from the membrane with SDS results in a denaturation and a loss of function of the polypeptides. Nevertheless, the positions of the five coupling factor subunits (107, 167, 221), cytochrome f (107), ferredoxin (109, 110), and plastocyanin (59) have been identified. In addition, the positions are known for a series of chlorophyll-protein complexes (27). The chlorophyll-proteins were designated as chlorophyllins by Thornber et al.(226), and these terms are used interchangeably in this thesis.

The chlorophyll-protein complexes, or

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chlorophyll-proteins, were first identified as green bands on SDS-PAGE gels by Chiba (50). Since that time, the preparation and purification of chlorphyllins have been refined to a level that allows the assignment of specific photochemical functions to the bands (see reviews 27, 223, 225). Until 1978, data on chlorophyll proteins from many laboratories described three pigmented bands on electrophoretic gels: a P700 chlorophyll a protein, thought to be the reaction centre of PSI; the light-harvesting chlorophyll a/b protein which has been proven not to be the reaction centre of PSII as previously believed (224); and a band of free chlorophyll running at the electrophoretic front. In a methodological study, Wessels and Borchert showed that lipid extraction, high detergent concentrations, or heating to 100C in SDS would cause the loss of chlorophyll from the chlorophyll protein complexes (241). By the use of lower detergent to chlorophyll ratios and less harsh extraction techniques, two additional chlorphyll a/b chlorophyll proteins were identified almost simultaneously in separate laboratories (153, 7, 99). These additional bands were thought to be dimers and trimers of the LHC. In addition, a unique chlorophyll a-containing band was found, similar to that first described by Hayden and Hopkins (94), which migrated between the LHC and the P700 chlorophyllin. This band was present in a chlorophyll b-deficient mutant of barley which lacks LHC but retains PSII activity (159), but was absent in a PSII-deficient mutant of barley (213). It

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has, therefore, been proposed that this band is the PSII reaction centre.

Machold et al. have described ten chlorophyll-containing bands plus free chlorophyll in wild-type barley (147). Broglie et al. used lithium dodecyl sulfate, rather than SDS, and obtained eleven chlorophyll-containing bands from Rhodopseudomonas (40). A novel extraction procedure involving SDS and a zwitterionic detergent, has been recently developed by Markwell et al and gel profiles were obtained with no free pigment band (154). These findings suggest that all the chlorophyll in the thylakoid membrane may, in fact, be bound to protein.

The site of synthesis of chloroplast proteins has been the subject of several recent reviews (67, 68, 76, 137). Based on analyses of purified chloroplast DNA, chloroplasts contain sufficient genetic information to code for an estimated 300-400 proteins of 20,000 d. (22, 96). The most abundant protein in the chloroplast, RUBP carboxylase, illustrates an interesting study of location and control of biosynthesis of a chloroplast protein. This protein is an aggregate of two types of polypeptide subunits (138). The larger subunit is coded and translated in the chloroplast, while the smaller one is coded in the nucleus and translated in the cytoplasm (137,138). This was initially hard to reconcile, as there was no satisfactory explanation of how a protein could cross a hydrophobic lipid membrane barrier. Ellis first proposed a mechanism involving a large precursor

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to the small subunit, which was shown to be transcribed by 80 s ribosomes *in vitro* (100). After this precursor was incubated with intact chloroplasts, the small subunit, but not the precursor, was found to have been transported into the plastids. The explanation of this phenomonon was that the "processing" of this precursor, or the removal of a small polypeptide chain at the chloroplast envelope was, in some way, linked to the transport of the protein across the membrane. The *in vitro* synthesis of higher molecular weight precursors of the LHC (53) and ferredoxin (111), two genomically-controlled proteins, supports the suggestion that this may be a universal mechanism for transmembrane protein transport.

RuBP carboxylase also provides a model with which to study the mechanisms that control the balance of protein synthesis in the chloroplast and in the cytoplasm so that an excess of one type of subunit is not produced. Two possible control mechanisms have been suggested by Ellis (67). The simplest mechanism assumes that the synthesis of the large subunit is controlled by a negative feedback of the large subunit, and that the small subunit acts like a derepressor by combining with the large subunit, effectively reducing its concentration. In this way an excess of the large subunit over the small subunit will repress the synthesis of the large subunit. The second mechanism assumes that the cytoplasmically synthesized small subunit is a promotor, and thus has a positive effect on the synthesis of the large

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subunit. At present, not enough is known to categorically support or reject either of these two proposals. The above discussion of RuBP carboxylase may be applicable to any organellar multimeric enzymes that have both plastome and genome contributions (52,67).

3. The Membrane System

As early as 1779, it was known that only the chlorophyll-containing portions of a plant were capable of photosynthesis (cf. 178). However, it was not until over a hundred years later that this function was localized to the chloroplasts (101). In those days only light microscopy was available and the plastid was seen as a chlorophyll-containing organelle, but only a limited amount of internal membrane structure could be discerned. With the advent of the electron microscope, the resolution of the chloroplast structure could be measured in nanometers. With this increased resolution, it was soon apparent that chloroplasts from different photosynthetic species can vary remarkably in morphology. By classical description, a chloroplast is a 5-10 um lens-shaped organelle surrounded by two limiting membranes which contains an internal lamellar region of stacked thylakoids, or grana (usually 4-10 thylakoids per granum), interconnected by stroma lamellae. This description, however, has many exceptions. The bundle-sheath chloroplasts of C4 species (140), as well as many algal species do not have stacked thylakoids (169). Algae of the Chromophyceae may have three or four limiting

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membranes (56, 63) and the shape of the chloroplast may be stellate as in Porphyridium (169), or helical as in Spirogyra rather than lens-shaped. Although the structural polymorphisms are great, the chloroplast function and the localization of specific activities are consistent throughout all photosynthetic species. For the purpose of simplification, the following discussion of chloroplast structure will be focussed on the classically described higher plant chloroplast.

The generation of ATP requires a charge separation which is maintained by a nonconductor- the lipid membrane. The dissipation of this electrochemical gradient results in ATP production. A charge separation by definition, requires an enclosed space or a sphere. In order to pack the maximum number of these enclosed spaces into the smallest possible volume, they should be collapsed, thus describing the structure of a single thylakoid. The "pile of pennies" structure of grana, then, is an efficient arrangement of the essential units required for ATP generation.

The development of the freeze-fracture technique of electronmicroscopy led to the characterization of the molecular architecture of chloroplast membranes (12). The method involves the quick freezing of an unfixed sample followed by sectioning with a "blunt" knife. Since the tissue is neither fixed nor embedded, the cutting is uneven and tends to follow the planes of least resistance which run through the hydrophobic regions of membranes. After

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preparing and shadowing a replica of the fractured face the internal substructure of the plastid membrane is revealed. The hydrophobic proteins deeply embedded in the lipid matrix of the bilayer are exposed, and appear as particles on the surface of the fracture face (reviewed by Staehelin 215). According to the recently adopted nomenclature, the two leaflets of the membrane are designated protoplasmic (PF) and exoplasmic (EF) faces (38). The P half of the membrane is that closest to the protoplasm or chloroplast stroma, and the E half is closest to the exoplasmic space or intrathylakoid space.

On the basis of freeze fracture particle sizes and densities (i.e. number of particles per unit area), Staehelin and coworkers were able to differentiate four characteristic faces in Chlamydomonas thylakoids (81). Two of these types were related to stacked regions or grana (EFs, PFs), and two to the unstacked regions or stroma lamellae (EFu, PFu). The EFs characteristically has a bimodal particle size distribution with modal means of 114A and 162A for spinach (214), 105A and 164A for pea (10) and 105A and 140A for Chlamydomonas (175). The PFs particles are small and fairly uniform in size with an average diameter of about 80A. The EFu is easily recognized because of its sparse population of particles that are the same size as the smaller size class of EFs particles. The PFu is the most densely packed fracture face and these particles fall into two size classes. Modal means are 82A and 118A for spinach

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(214), 70A and 105A for pea (10) and 70A and 105A for Chlamydomonas (175).

The mobility of the membrane particles within the plane of the membrane is apparent, since artificial destacking of spinach thylakoids, induced by low ionic strength buffers, leads to a total randomization of the particles within 45 minutes at 4C such that only two fracture faces- EF and PF- can be distinguished (214). This process is totally reversible upon readdition of cations to the medium. Staehelin and coworkers studied the kinetics of restacking and concluded that it occurs in two steps: a rapid appression of the thylakoid membranes, followed by a slow diffusion of particles into the stacked region (214).

Armond et al. (10) studied the freeze fracture particle size and density as a function of greening and found quantum increases in the EF particle sizes with greening time. These increases in size were considered to be related to increases in the amount of LHC and they suggested that this complex was in fact, being systematically added to the photosystem core. This interpretation is supported by the fact that a chlorophyll b-deficient mutant of barley (159), dark-grown Euglena cells (176) and the red alga Spermothamnion (215), all of which lack LHC, have generally smaller sizes of EF-particles.

Membrane fractionation studies have led to the conclusion that the EF particles are associated with PSII activity (13, 215), whereas the PF particles are associated

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with PSI activity (12,13). Radunz and coworkers have been able to chemically discern the position of many of the protein and lipid components involved in the PET chain by the use of specific antibodies (135, 182-186, 199-202). On the basis of these studies and with the prior knowledge that the result of PET is to generate an electrochemical gradient of protons, models of the thylakoid membrane have been proposed indicating the precise positions of the PET chain components within the membrane (91, 227, 245). On the basis of freeze-fracture data, models have been proposed indicating positions of photosystem particles within the membrane (10, 214). However, no one has yet combined the two types of information to generate a useful model relating the positions of the proteins within the thylakoids to the freeze-fracture particles in the chloroplast membranes.

B. Chloroplast Function

1. The Dark Reactions

The principle function of chloroplasts in green tissue is to reduce atmospheric carbon dioxide into metabolizable carbohydrates. This process makes photosynthetic organisms unique from eutrophic organisms and they are therefore referred to as primary producers. The absorption of light and the generation of ATP and NADPH, or the light reactions, are primarily associated with the membrane system and will be discussed in detail later. All the other reactions that occur in the chloroplast are called the dark reactions and

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are carried out in the plastid stroma.

The Calvin-Benson cycle is the ultimate pathway of carbon assimilation (234). It is unusual in that it is autocatalytic and can be summarized:

$$3CO_2 + 5H_2O + 9ATP + 6NADP \longrightarrow 1TP + 9ADP + 8P_i + 6NADPH$$

The autocatalytic effect is seen when the produced triose phosphate (TP) is fed back into the cycle, rather than being exported from the plastid. This would result in a theoretical doubling of the receptor for CO2 for every fifteen molecules of CO2 fixed (236). At steady state, the produced triose phosphate would be accumulated as starch or exported from the plastid to provide energy for the rest of the cell. The details of these reactions i.e. rate limiting steps, control points, cofactors involved etc. have been studied and reviews can be found by Walker (235), Preiss (181), and Bassham (16).

Chloroplasts can undergo protein synthesis independent of the nucleus in another "dark reaction" (for reviews see Ellis 67,68, Gillham, 76, Kung, 137). The mechanism of transcription and translation are much more akin to bacterial protein synthesis than to that of the eukaryotes. Chloroplast-messages are transcribed on 70S ribosomes which have rRNA complements similar to that of E. coli. In addition, chloroplast protein synthesis is sensitive to chloramphenicol and insensitive to cycloheximide. The near autonomy of this organelle is suggested by the fact that the

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ribosomal proteins, rRNAs, t-RNAs, cofactors, amino acids and other constituents of protein synthesis are mostly made within the chloroplast.

Many other reactions operate in the chloroplast such as sulfate reduction (203), nitrate reduction (144) and lipid biosynthesis (77,219). The transcripts for the enzymes of these pathways are encoded in both the nucleus and the chloroplast. Therefore, the communication between chloroplast and cytoplasm must be highly refined and the chloroplast envelope itself, an important control barrier.

2. Photosystem II

The reaction centre of PSII in higher plants and algae includes a specific form of chlorophyll with an absorption of 680nm (42). The light-stimulated excitation of this chlorophyll and subsequent transfer of electrons down the PET chain results in a "hole" for electrons which is refilled by electrons from a reaction associated with the oxidation of water.

The Hill reaction, or the splitting of water, has been under investigation for many years and yet less is known about this reaction sequence than any other in the PET chain. Manganese (49) and chloride (114) have been shown to be absolute requirements for water splitting and impart a high degree of lability for this portion of the PET chain. In fact, the loss of O2-evolving activity upon washing the membranes with TRIS buffer is thought to be due to the removal of Mn^{2+}, since the readdition of Mn^{2+} results in the

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reactivation of the Hill Reaction (24, 249). Although the O2-evolving mechanism is easily lost in isolated chloroplasts, the actual PSII trap and subsequent reactions may still remain intact. The stability of this latter part of the system is demonstrated with the use of artificial electron donors to PSII such as DPC, phenylenediamines, benzadine, catechol or hydroxylamine (93).

In 1955, Allen and Franck (2) recognized that a single 1 msec flash of saturating light did not result in O2 evolution if algae had been dark-adapted. This was the first piece of evidence to suggest that the O2-evolving system of PSII needed to be "primed" or that more than one "photoact" was involved. To further characterize this phenomenon, Joliot constructed a very sensitive polarographic apparatus, with which he could readily obtain flash yield data (119). The theory behind his experiments was that a single saturating short pulse would excite all traps once so that events could be followed stepwise and in synchrony. In all photosynthetic material used qualitatively similar results were obtained: no O2 evolution could be detected after the first two flashes and subsequent flashes led to a damped oscillation with a period of four (see reviews 141, 189). That is, maximum yields occur on flash numbers 3, 7, 11 etc. The interpretation of these data is that the splitting water involves four successive photochemical events, or the accumulation of four positive charges (136).

S0---->S1---->S2---->S3---->S4

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$$2\ H_2O + S4 \longrightarrow S0 + O_2 + 4H^+$$

Both S0 and S1 are dark stable, whereas S2 and S3 decay to lower oxidation states within minutes. Thus, after a period of dark adaptation, most of the centres will be in S1 and a maximum yield of O2 evolution will occur on the third flash. The damped oscillation was explained by assuming that a certain proportion of centres will undergo a double transition or a "double hit", while others will undergo no transitions or "misses". This assumption was supported by the facts that the proportion of double hits increased when the flash duration increased, and a more rapid dampening of the oscillation was seen (141). Since the original work of Joliot, Kok and coworkers, further evidence to support this theory has accumulated from experiments involving delayed light emission, fluorescence emission and other techniques (120).

NMR spectroscopy has indicated a direct correlation between the 1/T2, or proton relaxation rates around the paramagnetic Mn^{2+}, with flash yield (reviewed by Govindjee et al. 84). That is, a damped oscillation in the 1/T2 is seen with a periodicity of four and maxima at 3, 7, 11 etc. The proton NMR 1/T2 pattern is lost in chloride-depleted chloroplasts but can be restored upon readdition of chloride or fluoride (84). This suggests that chloride may be required as a ligand to manganese to help stabilize the higher oxidation states of Mn.

The energy required to generate S4 and result in

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water-splitting is provided by a "sink" for electrons at the oxidized chlorophyll which loses electrons in the photoact. An intermediate, Z, has been postulated to exist between the charge-accumulating Mn-protein and the chlorophyll of the PSII trap (46, 83). Although the chemical nature of Z is unknown, its existence is suggested by a fast-decaying, free-radical species detected by EPR spectroscopy and known as Signal II (14). The decay kinetics of signal II were greatly accelerated by lipophilic electron donors to PSII suggesting that Z may be the point of entry of these donors (15).

The PSII reaction centre involves a unique chlorophyll a, with an absorption maximum of 680 nm. The first evidence for this came from fast-absorption spectrophotometric measurements (73). A short laser flash could induce bleaching at 680 nm at 77K. This was thought to be due to the photooxidation of the reaction centre, resulting in a disappearance of the chlorophyll band at 680 and the appearance of a new band at 820, corresponding to the radical cation of chlorophyll a (55).

The primary acceptor of electrons from P680 has been designated Q because of its ability to quench the variable fluorescence of chlorophyll a. This fact was first recognized by Kautsky et al. (131). Knaff and Arnon (134) discovered a rapid light-induced absorption change at 550 nm, designated it C550, and claimed that this signal was caused by the primary acceptor of PSII. Subsequent evidence

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pointed to the equivalence of the C550 signal measured by absorption and the Q signal measured by fluorescence (see reviews 43, 121, 157).

The electron flow through PSII can be measured directly by observing the kinetics of chlorophyll a fluorescence (see review 44, 177). Much information can be obtained about the pool size of Q by the fluorescence induction curve upon the onset of illumination. The fluorescence represents the re-emission of light energy from the light-induced excited state of P680. If the excited state of P680 can transfer its excitation energy to Q^-, no chlorophyll fluorescence will result. On the other hand, if the energy transfer is blocked (i.e. by Q^---->Q) then some of the absorbed radiation will be re-emitted as fluorescence. Thus, if one includes the inhibitor DCMU in the reaction media, upon illumination all the traps will be closed, Q^- will be fully reduced, and there will be a sharp rise in the induction curve to a maximum (fmax). In the absence of DCMU, chloroplasts will exhibit a slower rise (4-5 sec.) which corresponds to the saturation of the PET system such that all the members of the PET are essentially reduced. The photosynthetic inhibitor DBMIB blocks the PET, not at Q as in the case of DCMU, but rather at the level of the second acceptor, plastoquinone. Thus, the area between the induction curves for DCMU and DBMIB inhibited chloroplasts should give an estimate of the pool size of the functioning PQ.

Plastoquinone A has been repeatably shown to be an

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intermediate between PSII and PSI, as it is reduced by PSII and oxidized by PSI (see reviews 4, 5). PQ has an absorption band at 260 nm and plastohydroquinone (reduced PQ) has a much weaker band at 290 nm (19). When chloroplasts are illuminated, a DCMU-sensitive decrease in the 260 nm band is seen (3). By analyzing the extent of these changes, Steil and Witt (218) were able to calculate that seven molecules of PQ were present in each PSII reaction centre. Bouges-Bocquet (32) and Velthys and Amesz (232) independently postulated the existence of another carrier designated as "B" or "R" respectively between Q and PQ to provide a mechanism by which the one-electron carrier (Q) can reduce a two-electron carrier (PQ). Evidence for the charge accumulation of "R" came from the fluorescence increase caused by a strong reductant (dithionite) as a function of the number of flashes before illumination (232). They found that the fluorescence yield oscillated with a periodicity of two and suggested a double-charge accumulation and transfer scheme, which has since been supported by other workers (65, 79, 88, 121, 194)

$$QR \text{----} > Q^{-}R \text{---} > QR^{-} \text{-----} > Q^{-}R^{-} \text{----} > QR^{2-},$$

$$PQ + QR^{2-} \text{----} > QR + PQ(H2)$$

A large number of oxidants such as quinones, indophenol dyes, ferricyanide and silicomolybdates have been used as artificial electron acceptors to measure PSII (see review 93). Most of these acceptors tend to pull out electrons at or near PQ. The indophenol dyes are not exclusively reduced

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by PSII and are, in fact, preferentially reduced by PSI. Reactions with these compounds are sensitive to DBMIB and thus accept electrons beyond the PQ pool. Benzoquinones and oxidized phenylenediamines are reduced by PSII in a DBMIB-insensitive reaction (230), but are sensitive to DCMU, identifying their point of removal as between Q and PQ. The PSII activity with SiMo is insensitive to both DBMIB and DCMU and it is thought that SiMo accepts electrons either directly from P680 or from Q (75).

3. Photosystem I

The PET chain and reactions of PSI are generally understood better than those of PSII. This is due, in part, to the inherent stability of PSI. The electron donors to PSI are essentially the acceptors of PSII, as the two photosystems act in concert. Cytochrome f and plastocyanin are two membrane-bound electron carriers that have been shown to be on the oxidizing side of PSI. Cytochrome f, a c-type cytochrome, and PC, a copper-containing protein, are present in thylakoids in equimolar ratios (26, 47, 57, 129). Studies of midpoint potentials (151, 190), ESR signal decay kinetics (89) and analysis of oxidation reduction in subchloroplast fragments (60, 90) and mutants lacking PC (209), indicate a flow of electrons from PQ through cytochrome f to PC and on to the PSI trap.

The principal electron donors to PSI used in artificial systems are reduced indolphenol dyes and substituted p-phenylenediamines. In both cases, the donors must be kept

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reduced by ascorbate to prevent the re-reduction of the oxidized form by PET components on the reducing side of the PSI trap. The substituted p-phenylenediamines are the most commonly used donors to PSI.

N,N,N¹,N¹-tetramethyl-p-phenylenediamine (TMPD) is not coupled, while 2,3,5,6-tetramethyl-p-phenylenediamine is coupled to phosphorylation (229) and the stoichiometry of the coupled compound is one half that of whole chain PET (227).

The first hints that a high wavelength-absorbing form of chlorophyll was the PSI trap came out of the work by Emerson and the "enhancement effect" (69). P700 exhibits most of the qualities of P680 except that it absorbs light at a slightly lower energy (see review by Hoch 104). The oxidation/reduction of P700 can be directly measured as the light minus dark, or reduced minus oxidized difference spectra, using a dual wavelength spectrophotometer (155). Katz has proposed a mechanism involving dimeric chlorophyll and a water ligand, which is photoconverted to a high energy chlorophyll radical (123, 130). Fong has suggested a similar model involving two water molecules (74).

The identity of the primary acceptor of PSI is a controversial point. The first identification of a compound whose oxidation kinetics correspond to the reduction kinetics of P700, was a spectroscopic component identified as P430 by Hiyama and Ke (102). This may, or may not, be a ferredoxin (103), as the extinction coefficient is

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considerably higher than any known for plant-type ferredoxins. Non-heme iron-sulfur centres have also been identified by Malkin and Bearden as early as 1971 (150). Since that time, a wealth of EPR data have suggested that two iron-sulfur centres are involved as primary acceptors of PSI (18, 78, 95, 207, 208). Ke has recently argued that based on half-reduction potentials, P430 and a bound iron-sulfur centre are identical (132). Indeed, Malkin has isolated and characterized an iron-sulfur protein which exhibits a photobleaching difference spectrum at about 430 nm (152). Thus, the non-heme iron-sulfur centre identified by EPR spectroscopy, and the P430 identified by absorption spectroscopy, may both be measurements of a unique type of membrane-bound ferredoxin which is the primary acceptor of PSI.

The final segment of PSI involves two soluble proteins - soluble ferredoxin and ferredoxin NADP oxidoreductase. The reactions of both of these proteins have been well characterized since, being soluble, they lend themselves to simple reconstitution experiments with the membrane bound segment of the PET chain (see review 92). Shin showed that this soluble ferredoxin is in an equimolar ratio with the reductase enzyme (206).

Many compounds can be reduced by electrons from the reducing side of PSI since the redox potentials of the membrane-bound iron sulfur centres are very low (70). Indophenol dyes and ferricyanide have been long used as PSI

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acceptors and their sensitivity to DBMIB (29) and dependence on long wavelength light indicate the primary point of acceptance is on the reducing side of the PSI trap. However, as mentioned before, care must be taken when using these compounds, as other points of electron donation and acceptance have been characterized. Low redox potential quinones such as anthroquinone and alkylated naphthaquinones and viologen dyes are more specific acceptors for PSI (93). The reduced forms of these compounds will undergo a spontaneous Mehler Reaction and the oxygen depletion can be easily measured with an oxygen electrode.

4. Cyclic Electron Transport

The PET scheme discussed above comprises the removal of electrons from water and their ultimate deposition to $NADP^+$. It is a one-way pathway catalyzed by the two photosystems and is referred to as noncyclic PET. The PET chain however, also exhibits the ability to function in a cyclical mode. The reaction sequence is thought to involve an additional b-type cytochrome, known as b6, with an absorption of 563 nm (57). This cytochrome is commonly extracted together with cytochrome f and may therefore be functionally, as well as structurally, related to cytochrome f (23).

Cyclic PET is a PSI-linked phenomenon since it is insensitive to DCMU and is active in far red light (210). The reaction is inhibited by DBMIB (125), antimycin (108) and DSPD (31). DBMIB-inhibition implicates the involvement of PQ, DSPD-inhibition implicates the involvement of

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ferredoxin and antimycin is a specific block of cyclic PET only. Thus, the pathway deduced from these inhibitor studies involves the normal noncyclic PET scheme from PQ to ferredoxin, but rather than transfering electrons to the $NADP^+$ reductase enzyme (i.e. if the enzyme is blocked by a build up of end products), the electrons are transferred to b6 and funneled back to PQ (212). The advantage of this cyclic electron flow is clear, since electrons flow back through PQ, a coupling site for phosphorylation. In other words, cyclic PET acts as a "clutch" that allows electron flow and the generation of ATP to continue even when large amounts of NADPH are present in the stroma.

5. Photophosphorylation

ATP, as well as reducing power, is required for the dark reactions of photosynthesis and chloroplast maintenence. There are two sites along the PET chain at which ATP synthesis is coupled to electron transport (115). The mechanism of coupling involves the generation of a proton gradient (the dissipation of which is linked to ATP synthesis) such as outlined in the chemiosmotic principle of Mitchell (162, 163). Although the chemiosmotic principle can be applied to all electron transport chains, the development of this idea stemmed from work on the PET chain. Jagendorf and coworkers first demonstrated a light-stimulated proton pumping into the thylakoids of spinach chloroplasts (168). Later, they showed that if a proton gradient could be artificially set up, phosphorylation would take place in the

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dark. This work was recently supported by Selman and Ort using spinach chloroplasts loaded with FeCN (204). The addition of a reduced lipophylic mediator (DAD) generated a proton gradient in the dark in a reaction insensitive to DCMU. This gradient was then dissipated with a concomitant formation of ATP. According to Mitchell, two energy parameters are involved in the proton gradient-the chemical gradient of protons (pH), and an electrical gradient set up by the charge separation (¬). These two parameters are additive, generating a proton-motive force which powers phosphorylation:

pmf = pH + .

The development of the proton gradient during PET has been explained by the spatial arrangement of the electron carriers across the membrane and by the inclusion of both proton and electron carriers in the PET sequence (227, 228). Models have been proposed describing the sites at which protons are taken up from the stroma in the reduction of $NADP^+$ and PQ. Protons are released into the thylakoids by the oxidation of water and PQH2 (82, 230). Thus, a net flow of protons from the stroma to the thylakoid space is seen (39).

A large protein, designated coupling Factor (CF1), composed of five different polypeptide subunits, is responsible for the transduction of the proton-motive force into ATP. These CF1 molecules are dotted over the surface of the thylakoid membrane and are attached to membrane proteins

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(CF0) which form proton channels through the membrane. Mechanisms for this reaction have been proposed by Mitchell (161), Boyer (34) and Williams (243), but, as yet, no conclusive evidence exists to prove any single proposal.

6. Excitation Energy Distribution

Since two photosystems exist in the PET chain, and each absorbs light at slightly different wavelengths, it is logical that a mechanism must exist to control the excitation energy distribution between the two photosystems, thereby optimizing the efficiency of the electron flow through the PET chain. The first evidence for this was provided by looking at low temperature fluorescence emission spectra (166). Fluorescence emission from the PSII pigment bed occurs at 685 nm, and from the PSI pigment bed at 734 nm (198). Murata noted that, on the addition of magnesium, an increase in PSII fluorescence at the expense of the PSI fluorescence, or a change in the 730/685 ratio, was seen in Porphyridium cruenatum (166). These data suggested that the efficiency of light trapping by PSII was increased. In other words, more of the excitation energy was being shunted to PSII in the presence of Mg^{2+}. The term "spillover" was coined to describe this intersystem energy transfer.

A tripartite model was proposed by Butler and Kitajima (47) which contained three chlorophyll domains: PSI units containing antennae chlorophyll and the PSI trap; PSII units containing antennae chlorophyll and the PSII trap; and the LHC which partitions the energy to either of the two traps.

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In a revised model, the PSI and PSII units are in contact with each other without the intermediary LHC (44, 45), which explains the phenomenon that spillover is unaffected in a mutant of barley which lacks the LHC.

Magnesium is known to induce major structural changes in thylakoid membranes, in that removal of Mg^{2+} causes a destacking of the grana (113, 175). This effect is fully reversible upon readdition of Mg^{2+}, and major rearrangements of the particles, as seen by freeze-fracture electron microscopy, occur in conjunction with the stacking and destacking (214). Removal of Mg^{2+} and subsequent destacking causes a shift in the excitation energy distribution to favor PSI (11, 166, 246). These findings suggest that the mechanism of excitation energy distribution is regulated by Mg^{2+} and involves the degree of stacking of the chloroplast thylakoids. However, no change in excitation energy distribution could be seen after artificially increasing the number of stacked thylakoids by centrifugation (216). Several lines of evidence now suggest that the LHC is responsible for the excitation energy distribution. The effect of Mg^{2+} is to regulate this via molecular changes in the PSII particles, too minute to be visualized by freeze-fracture electronmicroscopy, that affect the relationship with their immediate neighbours i.e. PSI particles (11, 41, 146, 216). The stacking effect then, may simply be a mechanism whereby components of the PET chain are concentrated to increase the overall efficiency of the

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system (156, 165).

C. Chloroplast Development

1. Structural Changes

The recent interest in the area of chloroplast development has generated the appearance of several reviews (35, 36, 48, 128, 142, 171, 179) and an international symposium in 1978. Most of the workers in this area have been characterizing the photomorphogenesis of angiosperm chloroplasts. There is general agreement that, under normal light conditions, the small proplastids of undifferentiated tissue will develop into chloroplasts as the tissue itself matures. Thus, in the meristematic, basal areas of a leaf of Zea mays for example, only proplastids are seen, whereas a few centimeters up into the green part of the leaf blade, fully mature chloroplasts are present (187). The study of greening under natural light conditions is confounded by the problem that plastid preparations are heterogeneous with respect to the developmental level of the chloroplasts. In order to synchronize the development of all the chloroplasts, plants can be grown in the dark to a certain stage and then placed in the light. This synchronization leads to more homogeneous preparations with respect to chloroplast development, which allows the study of the appearance of various parameters associated with greening. The major difficulty with this procedure is that when angiosperm seedings are allowed to develop in the dark, the

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proplastid develops into a "resting stage" -the etioplast. The etioplast is characterized by the presence of an internal structure of paracrystalline arrays or prolamellar bodies (143, 239, 248). Upon illumination, these structures appear to be the source of material for the rapid proliferation of thylakoid membranes. This artificial resting stage is never seen under normal conditions of chloroplast development. Therefore, the relevance of the etioplast to chloroplast transformation may be questioned.

Dark-grown gymnosperm seedlings synthesize chlorophyll, but do not develop photosynthetically competent chloroplasts until illuminated (231). Algal cells can be "degreened" by organotrophic growth in the dark for several generations and then synchronous chloroplast development can be started by illumination (171). In both of these lower plant groups the characteristic angiosperm etioplast is not seen in the absence of light. Rather, a structure, somewhat more developed than a proplastid is seen, which contains rudimentary thylakoids and in some cases, a prolamellar body.

Using either the algal (33) or the angiosperm system (54, 106, 118, 170), many membrane proteins separated by SDS-PAGE can be seen to either increase or decrease in abundance during greening. Bogorad and coworkers have compared the proteins synthesized by isolated plastids to those synthesized by greening maize seedlings (87). They found that most of the proteins present in the chloroplast

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and absent in the etioplast, are not synthesized by isolated plastids. From this, they concluded that most of the proteins made by the chloroplast are not tightly regulated by light. The photoinduction of two major thylakoid polypeptides - the 32 Kd "photogene" product (86) and the LHC (87) have been studied in detail, however, the mechanism of the photoinduction itself has yet to be realized.

Arntzen and coworkers have studied changes in the freeze-fracture particle sizes and distributions as a function of greening (10). Pea seedlings, allowed to green under an intermittent light regime, produced plastids with full photochemical competence, but which lacked LHC and grana (9, 58). Freeze-fracture faces of thylakoids from these plants exhibited EF and PF particles, which were small and uniform in size (80A and 70A respectively). After continuous illumination and subsequent insertion of the LHC into the membrane, additional EFs size classes sequentially appeared at 105A, 132A and 164A. After 48 h in the continuous light, only the 132A and 164A size classes were seen. The increases in particle volume associated with these increases in diameter were compared to an estimated volume of the LHC. It was concluded that the 132A and 164A particles consisted of a core complex with two and four LHC subunits respectively added to it.

Proplastids and etioplasts are incapable of carrying out photosynthesis. The first event in the development of a photosynthetically competent chloroplast is the

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photoconversion of protochlorophyll to chlorophyll. This interconversion, which is accompanied by an in vivo absorption maximum change from 684 nm to 672 nm, was first described by Shibata, and is referred to as the Shibata shift (205). Chlorophyll a is synthesized before chlorophyll b and therefore, the chlorophyll a/b ratio immediately after the onset of illumination is very high. During the first few hours of greening, the chlorophyll a/b ratio drops to a stable level concomitant with the thylakoid membrane proliferation and grana stacking (66, 98, 180, 240, 242). Thus, an inverse relationship exists between the chlorophyll a/b ratio and the degree of membrane stacking. The development of cation-induced stacking capacity of pea thylakoids parallels the appearance of chlorophyll b (8). This, added to the findings of Arntzen and coworkers, supports the view that the LHC-protein is responsible for membrane stacking (156, 165).

P700 was absent during the first 30 minutes of greening in Chlorella, but a rapid production of P700 occurred over the next hour (66, 242). Cytochromes f, b6 and the low potential form of b559 as well as plastocyanin are present in barley etioplasts (98, 180) and their levels do not change significantly during greening. In Chlorella however, the cytochrome f content increases 3-fold during greening (242).

2. Functional Changes

The time course of development of the photochemical

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activities in chloroplasts varies markedly with the age of the seedlings, species of plants, light intensity and temperature, therefore it is difficult to quantitatively compare results from different laboratories. Qualitatively however, PSI activity appears to develop before PSII, and it is the water-splitting Hill reaction that is the last sequence to become active. The development of PSI activity was detected within 15 minutes of illumination in oat seedlings, when DAD was used as an electron donor but not until 30 minutes or 1 hour when TMPD or DPIP respectively were used as donors (240). Although the kinetics of development of PSI and the appearance of P700 are similar, there is some evidence to suggest that PSI activity may precede the appearance of P700 (66, 164, 180). Since the PSI activity is apparent when the chlorophyll content is very low, the PSI activity expressed on a unit chlorophyll basis, has a maximum within 2 to 3 hours from the onset of illumination. This peak may be as much as five to ten times higher than the rate of mature plastids. When expressed on a protein or fresh weight bases, however, a steady increase in activity is seen for about 8 to 10 hours (180).

The development of active PSII centres has been studied primarily by the use of fluorescence induction (1, 48, 62, 64). Cahen et al. (48) have characterized two phases in development in Chlamydomonas. Immediately after illumination, a rapid reorganization and connection between preexisting components occurs. This is followed by a phase

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of accumulation of newly formed, complete, and active units. Other authors have also suggested that the active centres of the PET chain are present at the onset of greening in Chlorella (97), Euglena (64) and Cyanidium (62) and that the initial photomorphogenesis is an induction of connectivity of the systems. If DPC is used as an artificial donor to PSII, the activity is apparent earlier in the greening process than if water is the donor (240). Thus, the PSII trap is in place and functional before the water splitting system becomes competent.

The last system of photosynthesis to develop is the proton pumping activity and the ability to undergo photophosphorylation (238). In Avena, the ability to carry out DAD-dependent proton pumping occurs after about 2 hours and DAD-dependent photophosphorylation is first seen after 3 hours of greening (240). Cyclic PET using PMS as a cofactor, has been shown to be active within 15 minutes of the onset of illumination (180, 240). This is not unexpected, since the PET chain components are already present at that time. The development of proton pumping and DAD-mediated photophosphorylation however, requires the presence of intact thylakoids to maintain the proton gradient. As a result, the time scale of the development of photophosphorylation closely parallels that of membrane proliferation and grana development.

The photosynthetic unit was originally conceived to be a physical description of the photosynthetic apparatus

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defining the number of chlorophyll molecules associated with the evolution of a molecule of oxygen. With the advent of freeze-fracture electronmicroscopy, it has become apparent that no structural entity can be seen that corresponds to the photosynthetic unit. Thus, its usefulness as a physical description is lost. The concept is useful however, when the photosynthetic unit is recognized as a statistical parameter of chlorophyll content. In this sense, a change in size of the photosynthetic unit with development is essentially a measure of changes in chlorophyll levels on a unit reaction centre basis. Different interpretations of the photosynthetic unit have led to results indicating an increase (97, 98), decrease (66, 242) or no change in size (17) during greening.

Several measurements can be made to detect changes in photosynthetic unit size during development. Photochemical activity on a unit chlorophyll basis during the period of increasing chlorophyll levels can be used. If no change in the size of the photosynthetic unit occurs during greening, then the activity /unit chlorophyll should remain constant. If the units are increasing in size, an initial peak of activity would be expected while the chlorophyll levels are low, followed by a decline to a stable level. The light saturation point, or amount of light required for maximal photosynthetic activity has been classically used when describing alterations in photosynthetic unit sizes. The rationale is that a larger photosynthetic unit has more

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chlorophyll/reaction centre, and, since the "target" is larger, less light is required for activity (97). If the photosynthetic unit size does not change with greening, the light saturation points will not change. Thus an increase or decrease in unit size should be reflected by a decrease or increase in the light saturation points respectively. Fleishaker and Senger (72) adopted a novel way of functionally defining the photosynthetic unit size based on the inhibition kinetics of DCMU. If one molecule of DCMU binds to one reaction centre, then the number of molecules of DCMU required to block the activity of a unit chlorophyll will describe the number of reaction centres per unit chlorophyll. This is, in fact, the inverse of the definition of photosynthetic unit size. Measurement of changes in size of photosynthetic units in developing chloroplasts are complicated by the de novo production of reaction centres, changes in connectivity of centres already present, and the development of stacked thylakoids during membrane proliferation.

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III. CHLOROPHYLL AND PROTEIN COMPONENTS OF PHOTOSYNTHETIC ELECTRON TRANSPORT

A. Materials and Methods

1. Plant Material

Gateway barley (Hordeum vulgare L. Ca. Gateway and its virescens mutant were used in this study. Throughout this thesis the wild-type or normal barley will be designated "N" and the virescens mutant "M". This mutant is phenotypically characterized by a seedling which is pale yellow-green upon emergence, but which later recovers and becomes a viable green plant (233,237). The mutation is a single gene nuclear mutation (217) and the lag in chlorophyll biosynthesis is affected by temperature and light intensity (160).

Seeds of the two lines were planted in trays of vermiculite and watered with a full-strength Hoagland's nutrient solution. For etiolation/greening studies, the plants were kept in a dark cabinet at 22°C for 6 or 8 days. Following the dark treatment, the seedlings were transferred to a light cabinet kept at 22C and illuminated at 8000 lux for various periods of time. Plants grown under continuous light for 6 or 8 days were also grown in these cabinets under the same conditions.

2. Chemicals

Acrylamide, bisacrylamide, TEMED and APS were all purchased from BIORAD Laboratories, Richmond, Ca. HEPES and Coomassie blue R-250 was from Sigma Chemical Co., P.O. box

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14508, St. Louis, Mo., and the SDS was from MCB Manufacturing Chemists Inc., Darmstadt, Germany. All other chemicals used were of reagent grade or better.

Molecular weight protein standards were obtained from BIORAD and consisted of lysozyme (14.3 Kd), soybean trypsin inhibitor (21 Kd), carbonic anhydrase (30 Kd), ovalbumin (43 Kd), BSA (68 Kd) and phosphorylase b (94 Kd).

3. Chloroplast Isolation

Fifty gm of leaf laminae (top 4 cm of the leaf) were cut into 1 cm sections and homogenized for 15 sec in a Waring blendor with 250 ml of 67 mM phosphate buffer (KH_2PO_4/Na_2HPO_4)(pH7.6 at 22C), containing 0.5 M sucrose, 1 mM $MgCl_2$ and 0.2% BSA. The homogenate was filtered through eight layers of cheesecloth and two layers of nylon mesh (50 um pore size), and centrifuged at 4000 g for two minutes (chloroplasts) or three minutes (etioplasts). The crude pellet was washed twice with a resuspending buffer consisting of 50 mM HEPES (pH7.6 at 22C); 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$ and 1 mM $MnCl_2$ (61). Any remaining intact chloroplasts were broken by osmotic shock in pipetting during the second wash. The entire isolation was carried out at 4C using cold glassware and was accomplished in less than 10 minutes from the initial grinding.

4. Plastid Counts, Chlorophyll and Protein Determinations

Plastid concentrations were calculated from counts made under a microscope using a haemocytometer.

Chlorophyll was extracted by grinding leaf tissue in a

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Ten Broeck homogenizer or suspending an aliquot of a chloroplast preparation in 80% acetone. This extract was then gravity filtered through glass wool and the resulting filtrate was taken to volume by the addition of more 80% acetone. For very pale chloroplast samples, one volume of the preparation was added to four volumes of 100% acetone and filtered. In all cases the absorption at 700 nm was subtracted from that at 663 nm and 645 nm before the calculations were made using the coeffecients of Mackinney (148).

The protein content of the samples was determined by the method of Bradford (37) using BSA (Sigma) as a standard.

5. Plastoquinone Analysis

Plastoquinone extraction and analysis was carried out according to the procedure of Barr and Crane (19). A chloroplast sample (10 ml) containing from 200-800 ug chlorophyll/ml was added to 100 ml of an isopropanol/heptane mixture (1:1) with 40 ml of deionized water in a 250 ml separatory funnel. This mixture was swirled and allowed to extract for 1 hr in the dark on a shaker. After separation, the aqueous phase was washed with an additional 50 ml of heptane and allowed to extract for 30 min. The aqueous phase was then washed a second time with a mixture of heptane, benzene, acetic acid and methanol (5:1:5:5). All the heptane extracts were pooled and washed twice with 50% methanol to remove the glycolipids. Sodium sulfate (5 g) was added to remove any water remaining in the samples and these heptane

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extracts were taken to dryness on a rotary evaporator.

The quinones were purified on small alumina columns (10x1.0 cm) plugged with glass wool. The column was prepared by vigorously mixing 33 g of acid-washed alumina (Merck) with 2.0 ml of distilled, deionized water, followed by 33 ml of petroleum ether. This slurry was added to the columns and they were immediately ready to use. The dried heptane extracts were disolved in 5.0 ml of petroleum ether and added to the columns. The first fraction, containing B-carotene and Vitamin K was washed off with 15 ml 0.2% diethyl ether in petroleum ether. The second fraction was eluted with 15 ml of 4% diethyl ether in petroleum ether and contained most of the plastoquinone. A third fraction was washed off with 12% diethyl ether in petroleum ether which contained predominantly tocopherols. The fourth fraction, containing the tocopherolquinones and plastoquinone C, was washed through with 20% diethyl ether in petroleum ether. The final green fraction eluted with 100% diethyl ether could never be completely removed from the columns but contained primarily chlorophyll and xanthophylls.

The purity of the collected fractions was characterized by thin layer chromatography on activated silica gel plates using chloroform/heptane (80:20) as the solvent system. Quinones were detected by spraying the plates with a reduced methylene blue spray and tocopherol by Emmerie-Engle Reagent (19).

For quantitation of the plastoquinone levels, fraction

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II from the alumina column was dried in a rotary evaporator and resuspended in 5.0 ml ethanol. This totally oxidized form of plastoquinone was placed in the reference cell of a dual beam spectrophotometer (Beckman Model 25). To the sample cell was added the same extract plus a few crystals of potassium borohydride to fully reduce the plastoquinone. After a few minutes, the reduced minus oxidized difference spectrum was scanned from 220 nm to 320 nm. The micromolar concentration of the plastoquinone was then calculated from the maximum absorbance difference seen at 255 nm divided by the millimolar extinction coefficient of 15 for plastoquinone (19).

6. Electrophoresis of Membrane Proteins

SDS-PAGE of membrane proteins was essentially carried out using the discontinuous buffer system originally described by Laemmli (139). Chloroplast membrane pellets were resuspended in Tris-buffer to give a final concentration of 0.625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue, with an SDS to chlorophyll ratio of 20:1. The samples were then immersed in boiling water for 5 min and centrifuged for 1 min in a Beckman microfuge. PAGE slabs (100 x 140 x 1.5 mm) were prepared using the apparatus of BIORAD. Both the running and stacking gels were prepared from a stock solution of 30% acrylamide and 0.8% bisacrylamide. The gels were polymerized using 0.025% TEMED and 0.1% APS. The 8% acrylamide running gel contained 0.375 M tris/HCl (pH 8.8)

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while the 3% acrylamide stacking gel contained 0.125 M tris/HCl (pH 6.8). Both gels contained 0.1% SDS. The electrode buffer was 0.025 M Tris/HCl (pH 8.3), 0.4 M glycine and 0.1% SDS. Gels were prerun for 30 minutes at 10 mA per slab before application of the sample (150 ug of protein per lane). The total running time at 10 mA/slab was from 5 to 6 hours.

After electrophoresis, the gels were removed from the apparatus, rinsed in distilled water, and stained overnight in 0.025% Coomassie Brilliant blue R250 in 25% isopropanol, and 10% acetic acid in water (71). Destaining was carried out in the isopropanol:acetic acid:water mixture in a BIORAD diffusion gel destainer.

7. Electrophoresis of Chlorophyll Proteins

The electrophoresis of chlorophyll proteins was carried out essentially by the method described above for membrane proteins, with a few modifications. The SDS:chlorophyll ratio was reduced to 10:1 and the gels were run in the dark at 5C. The samples were not boiled in the SDS, but allowed to extract for 30 minutes in the dark at 5C before centrifugation and electrophoresis.

8. Sample Preparation and Scanning Electronmicroscopy

For SEM it was essential to prepare intact chloroplasts, therefore the standard isolation procedure was modified slightly. The leaf laminae were chopped very finely with razor blades before grinding in a Waring blendor. Grinding was accomplished by only two short bursts (<1sec)

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of the Waring blendor. This led to a low recovery, but the plastids obtained had a high degree of intactness as indicated by phase contrast microscopy. After centrifugation the pellets were resuspended by gentle agitation with a camel's hair brush. The washed chloroplast pellet was resuspended in 5% glutaraldehyde in the resuspension buffer (50 mM HEPES, pH 7.6; 0.33 M sorbitol; 2 mM EDTA; 1 mM MgCl2; 1 nM MnCl2) for 15 min at 5C. The fixed chloroplasts were centrifuged at 2000g for 2 min and washed once with the resuspending buffer. The fixed, washed pellet was then postfixed with 1% OsO4 in resuspension buffer for 15 min at 5C. The plastids were once again spun down, washed in resuspending buffer, dehydrated in a graded acetone series followed by isoamyl alcohol and critical point dried before observation with a Cambridge Scientific S4 Stereoscan SEM.

9. Transmission Electronmicroscopy - Thin Sections

Leaf laminae were cut into 1 mm^2 sections in cold 2% glutaraldehyde and fixation was continued in vacuo for 1 hr at 0C. The samples were then washed three times in cold 67 mM phosphate buffer (KH_2PO_4/Na_2HPO_4) (pH 7.6) containing 0.5 M sucrose. Post-fixation with 1% OsO4 was carried out in vacuo at 0C for 1 hr and the tissues were again washed three times with cold buffered sucrose. Tissue was dehydrated in a graded acetone series, transferred to propylene oxide and embedded in Epon 812. Thin sections were cut with a glass knife, stained with 5% uranyl acetate for 1 hr followed by Reynold's lead citrate for 4 min, and examined with a

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Phillips EM 300.

B. Results

1. Chlorophyll Changes Associated with Greening

The changes in the chlorophyll a/b ratio and total chlorophyll/gram fresh weight in both M and N preparations as a function of greening are shown in Figures 1 and 2. There was a two hour lag in the drop of the chlorophyll a/b ratio in the M compared to the N after 6 days of etiolation. After 8 days of etiolation it took a longer illumination time for the chlorophyll a/b ratios to drop in both N and M chloroplasts. The chlorophyll a/b ratios were not reproducible in the early stages of greening because of the extremely low chlorophyll content in the isolated chloroplast membranes. The chlorophyll a/b ratios of seedlings grown under continuous illumination for 6 or 8 days were significantly lower in the M. The changes in the chlorophyll content of seedings on a fresh weight basis indicates that although the chlorophyll content of the N is always greater than that of the M, after 8 days of dark pretreatment, this difference was markedly reduced.

In another experiment, the seedlings were kept etiolated for various periods of time, and the rate of greening, measured as chlorophyll content after 24 hr in the light, was compared for the N and M seedlings. The most rapid rate of chlorophyll accumulation in both normal and mutant genotypes occurred after 6 days of dark pretreatment

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Fig. 1

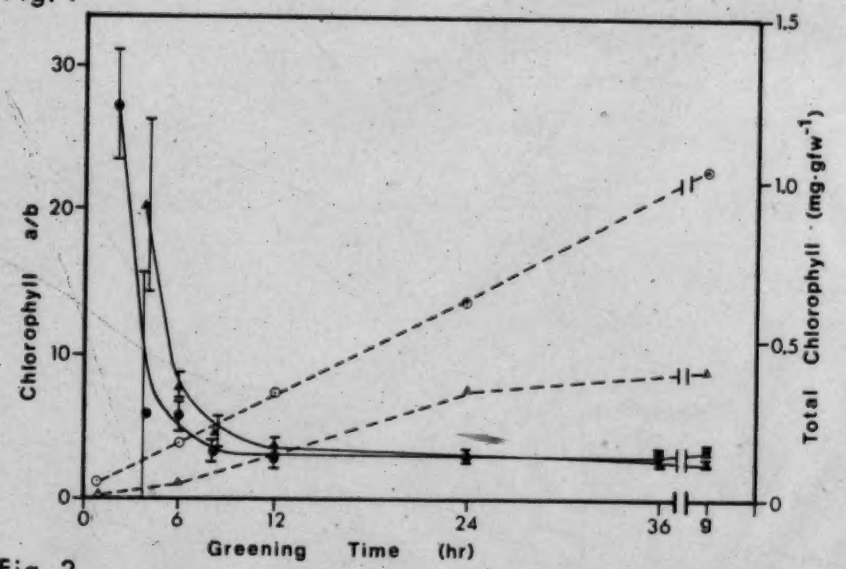

Fig. 2

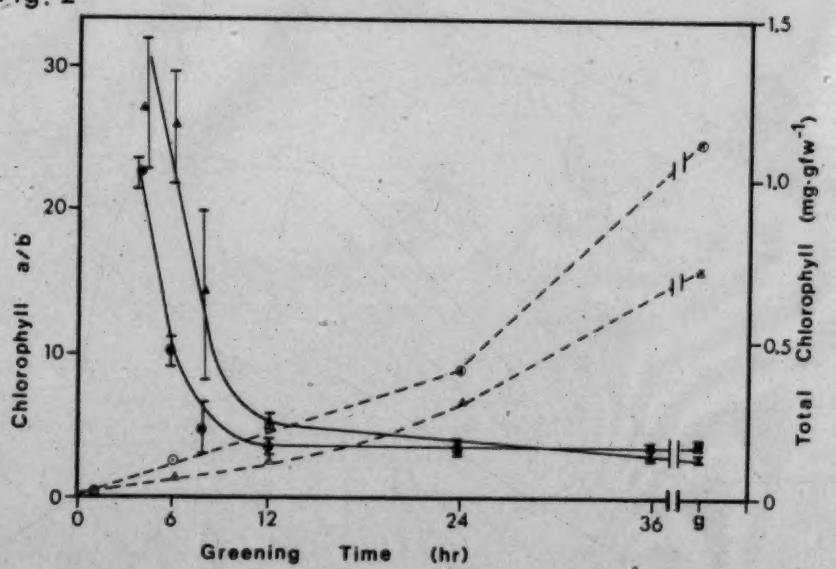

Fig. 1. Chlorophyll accumulation during greening in 6 day etiolated normal and mutant seedlings. Chlorophyll content in normal (○) and mutant (△) seedlings is given on a gram fresh weight basis. (●), chlorophyll a/b for normal; (▲), chlorophyll a/b for mutant tissue; g, values for seedlings grown for 6 days under continuous illumination. The values plotted are the means of eight replicates. Bars = S.E.

Fig. 2. Chlorophyll accumulation during greening in 8 day etiolated normal and mutant seedlings. Chlorophyll content in normal (○) and mutant (△) seedlings is given on a gram fresh weight basis. (●), chlorophyll a/b for normal; (▲), chlorophyll a/b for mutant tissue; g, values for seedlings grown for 8 days under continuous illumination. The values plotted are the means of eight replicates. Bars = S.E.

(Fig. 3). After 8 days in the dark, the greening rates of the N and M were similar. This lack of difference after long periods of etiolation, appeared to be due to a more rapid decline in the greening rate of N tissues rather than to changes in M tissues.

The protein and chlorophyll content on a plastid basis are shown in Figures 4 and 5 for chloroplasts from N and M seedlings. This index of change in chlorophyll levels should be more accurate than the fresh weight basis since variations in the leaf water content during greening would result in artificial fluctuations in the chlorophyll content. On a qualitative basis however, both sets of curves for chlorophyll accumulation (Fig. 1 and 2 and Fig. 4 and 5) were quite similar. Mutant seedlings greened for 24 hr, following 6 days of etiolation had a chlorophyll content of 51% of the N on a plastid basis, and 58% on a fresh weight basis. After 8 days of etiolation these values had increased to 72% and 71% respectively. The total protein levels per plastid did not appear to change extensively throughout greening. After 6 days of etiolation the protein content of the M etioplasts was 76% that of the N, but after 8 days this value had risen to 88%. Following 36 hr of illumination however, the M appeared to have a higher protein content than the N measured on a plastid basis.

2. Plastoquinone Analysis

a) TLC of purified fractions. Fractions I to IV from the alumina columns were analyzed by TLC on silica gel

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Fig. 3

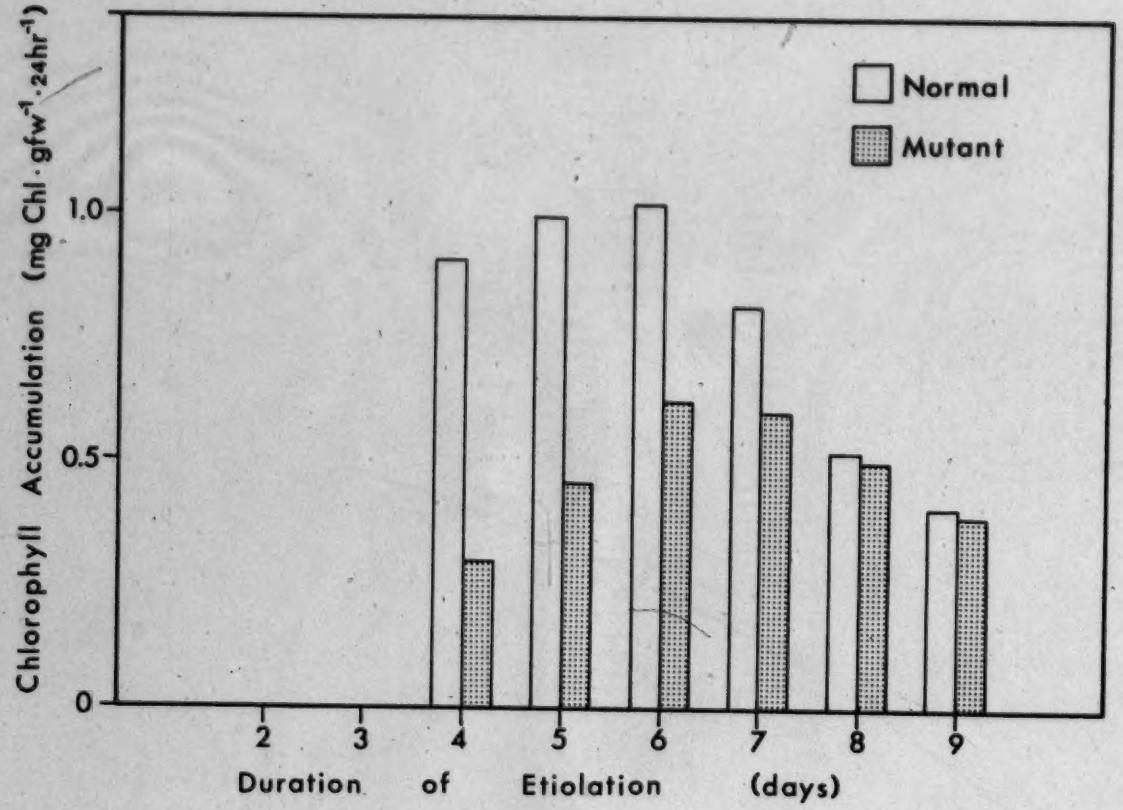

Fig. 3. Chlorophyll accumulation in the first 24 hr of greening in normal and mutant seedlings etiolated for various lengths of time.

Fig. 4

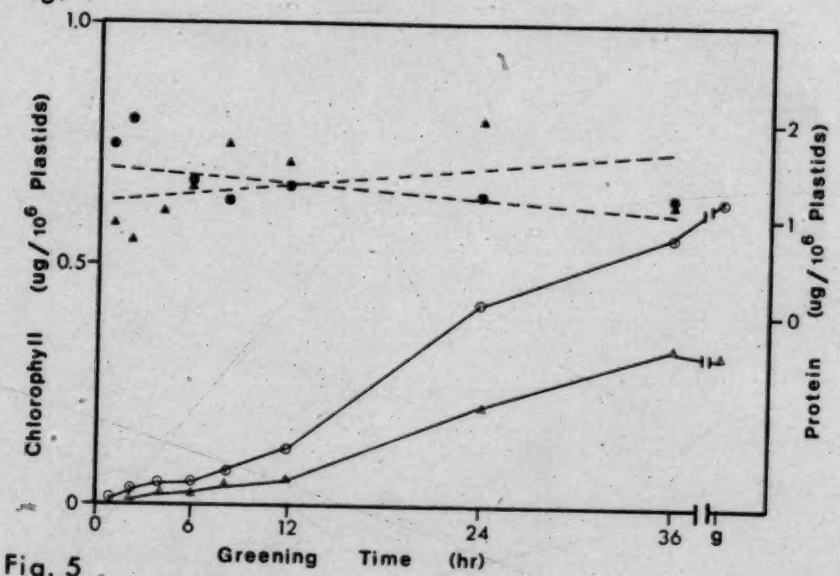

Fig. 5

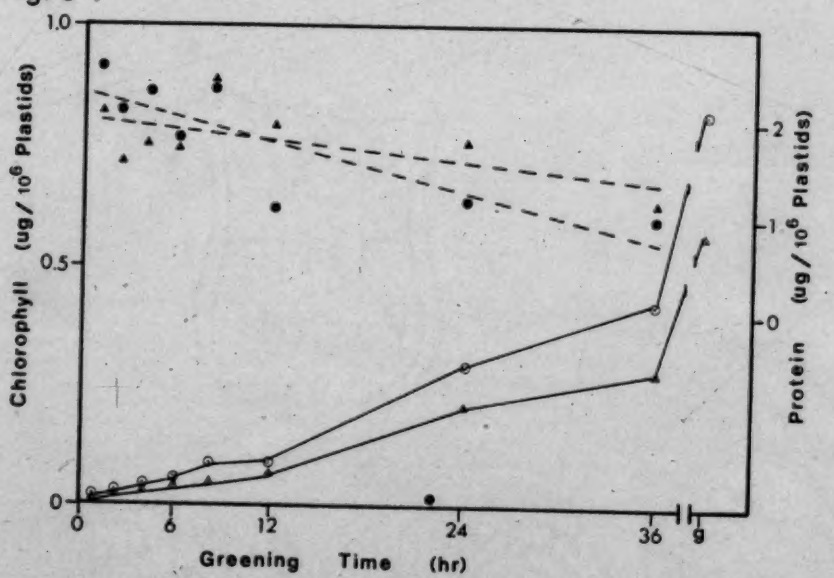

Fig. 4. Accumulation of chlorophyll and protein on a plastid basis in 6 day etiolated normal and mutant seedlings. Chlorophyll content in normal (⊙) and mutant (△) and protein content in normal (●) and mutant (▲) are given on a per million plastid basis. g, values for seedlings grown under continuous illumination. The values plotted are the means of four replicates.

Fig. 5. Accumulation of chlorophyll and protein on a plastid basis in 8 day etiolated normal and mutant seedlings. Chlorophyll content in normal (⊙) and mutant (△) and protein content in normal (●) and mutant (▲) are given on a per million plastid basis. g, values for seedlings grown under continuous illumination. The values plotted are the means of four replicates.

plates in 80:20 chloroform/heptane. A diagramatic representation of these fractions is shown in Fig. 6. Fraction I appeared to be primarily B-carotene, but a minor spot to a position corresponding to Vitamin K (19) appeared 30 min after spraying the plates with reduced methylene blue reagent. Fraction II was fairly clean with only one spot migrating to a position corresponding to plastoquinone A and which appeared bright blue immediately after spraying the plate with reduced methylene blue reagent (19). Little could be seen in fraction III other than two very faint red spots which appeared after spraying with Emmerie-Engel Reagent. The spots in fraction III were tentatively identified as tocopherols. Fraction IV had several spots that appeared upon spraying with reduced methylene blue reagent. The most prominent ones correspond in positions to tocopherolquinones and a minor spot higher on the plates corresponded to plastoquinone C (19). On the basis of these results, Fraction II only was used for the quantitation of plastoquinone.

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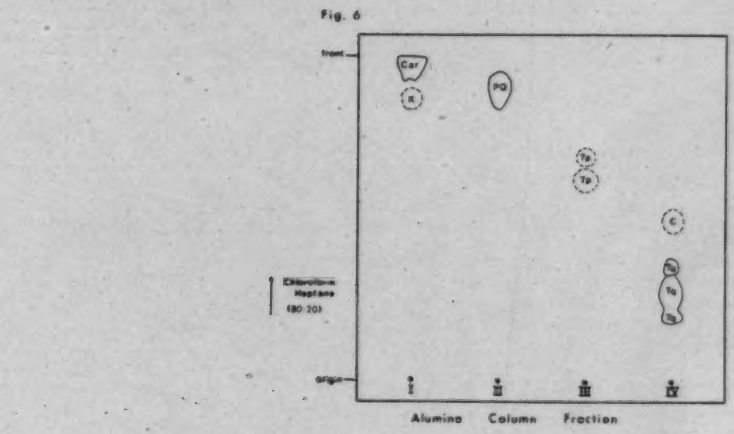
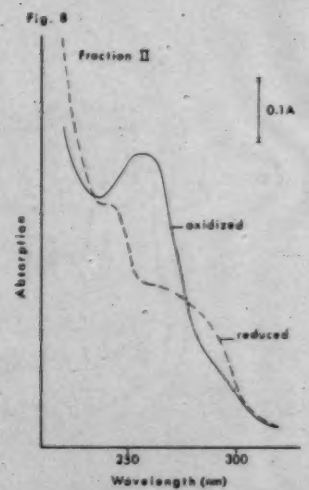
b) Spectral characteristics of Purified Fractions. The absorption spectrum of fraction I is shown in Fig 7. This spectrum was unaffected by addition of sodium borohydride and was essentially identical to that of B-carotene in EtOH, having peaks at 451 nm and 475 nm and a shoulder at 425 nm (80). The absorption spectra of the oxidized and borohydride-reduced forms of fraction II against an ETOH blank are shown in Fig. 8. The oxidized minus reduced





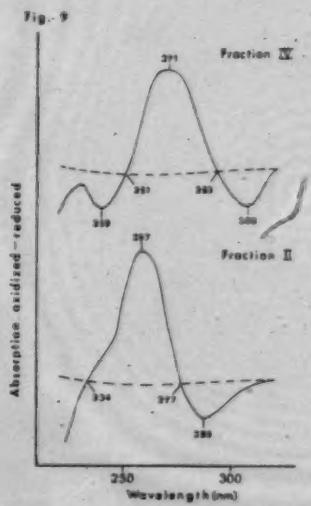


Fig. 6. Thin layer chromatogram of the four quinone fractions extracted from the alumina column. Car, B-carotene; K, vitamin K, PQ, plastoquinone A; Tp, tocopherols; C, plastoquinone C; Tq, tocopherolquinones.

Fig. 7. Absorption spectrum of quinone fraction I.

Fig. 8. Absorption spectra of oxidized and reduced quinone fraction II. Fractions were scanned against an ethanol blank. The oxidized extract was reduced by the addition of a few crystals of potassium borohydride 5 min before scanning.

Fig. 9. Oxidized minus reduced spectrum of quinone fractions II and IV. The oxidized sample was scanned with an identical sample in the reference cell to produce a baseline. The reference cell sample was then reduced by the addition of potassium borohydride and the rescan generated an oxidized minus reduced difference spectrum.

difference spectrum is shown in Fig. 9. The maximum difference was seen at 257 nm and the minimum at 288 nm. Isobestic points were at 277 nm and 234 nm. All of these data concur with those previously reported for plastoquinone A (19). The oxidized minus reduced difference spectrum for fraction IV is shown in Fig. 9. The maximum difference was seen at 271 nm and two minima were seen at 239 nm and 308 nm. The isobeistic points were at 251 nm and 293 nm. Based on spectral and TLC results, fraction IV was identified as a mixture of tocopherolquinones and plastoquinone C.

c) Quantitation of plastoquinone Levels in Chloroplast Preparations. The plastoquinone A levels were determined from the oxidized minus reduced difference spectra for plants grown under continuous illumination. The results are shown in Table I. There was a significantly higher level of plastoquinone A on a unit chlorophyll basis in the M chloroplasts after both 6 and 8 days of continuous illumination. When converted to a tissue fresh weight basis however, the N leaves had about twice the plastoquinone A content of the M at 6 days, whereas after 8 days, the values were almost equal. The chlorophyll/plastoquinone molar ratio, however, is the functionally significant parameter. These ratios indicate an abundance of plastoquinone A with respect to chlorophyll in the M at both 6 and 8 days. Developmentally, the older tissues had higher plastoquinone A levels and the increases from 6 to 8 days were fairly consistent in both N and M chloroplasts.

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Table I. Extractable plastoquinone A levels in chloroplasts from N and M seedlings grown for 6 or 8 days under continuous light.

Sample	plastoquinone A		
	uM/mg chl	uM/gfw	Chl:PQ**
6 day N	0.025 ± .004*	29.0	45
6 day M	0.036 ± .006	14.5	33
8 day N	0.031 ± .002	35.0	37
8 day M	0.045 ± .002	33.1	25

* values given are means ± 1 S.E. from five separate extractions

**Chl:PQ molar ratios were calculated from the uM/mg chl values, using a value of 1.1 umol chl/mg chl.

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3. Electrophoresis of Membrane Proteins

SDS-PAGE gels of thylakoid membrane proteins from N and M seedlings grown under continuous illumination for 6 and 8 days are shown in Fig. 10. Equal amounts of protein were loaded on the gels, and therefore the N samples had much more chlorophyll loaded than the M. Nevertheless, the gel patterns from all of these samples were similar.

The polypeptide profiles changed markedly as a function of greening as seen for the 6 day etiolated plants of the N genotype (Fig 11). Some protein bands were lost, and others appeared during greening. It must be remembered however, that these changes were relative changes; a simple decrease in staining intensity of a certain protein band on the gel need not necessarily mean that its absolute value was declining. It simply means that its concentration, relative to the rest of the proteins in the sample was decreasing. A general trend can be seen in the disappearance of the very high and very low molecular weight proteins as a function of greening. Specifically, bands at 96 Kd and 66 Kd, as well as several below 20 Kd showed a characteristic decrease during greening. A diffuse band at 38 Kd in the early stages of development, split into a doublet at 38 Kd and 40 Kd in the later stages. A 34 Kd band showed significant increases in the later stages of greening, although it was present in low amounts even after 2 hr in the light. The most obvious change was the development of a doublet around 29 Kd which, in green tissue, was perhaps the major protein band. Other

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Fig. 10

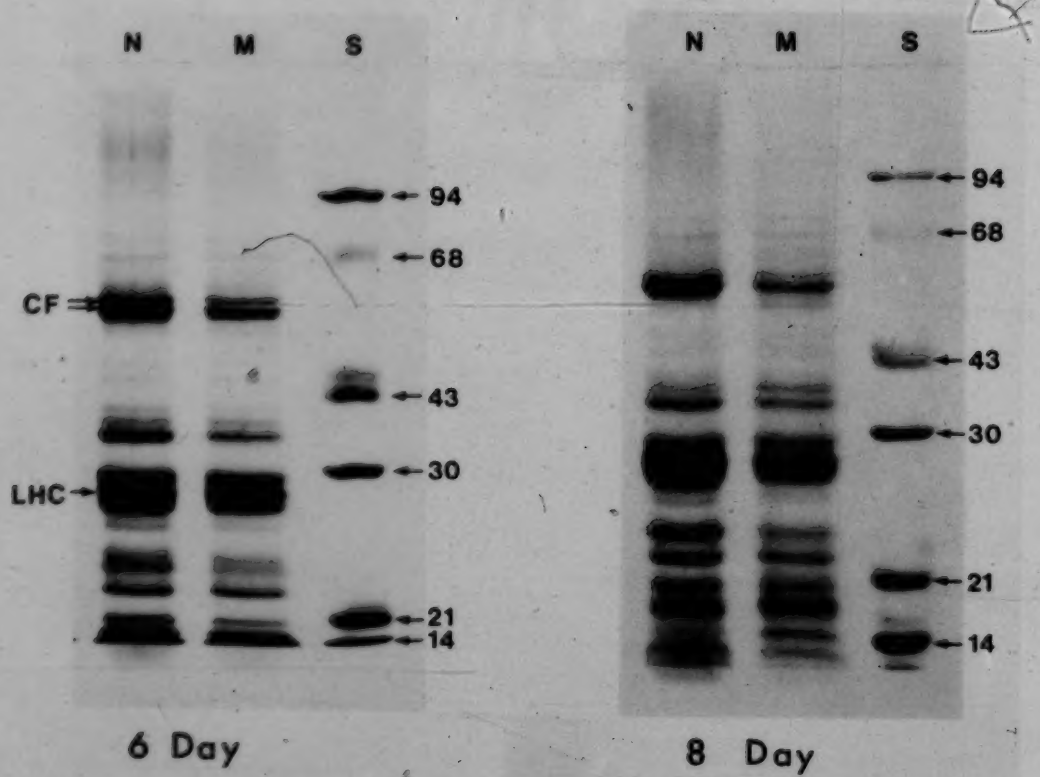

Fig. 10. SDS-PAGE of thylakoid membrane proteins from normal and mutant chloroplasts grown for 6 or 8 days under continuous light. N, normal; M, mutant; S, protein molecular weight standards; CF, coupling factor; LHC; light harvesting complex.

Fig. 11

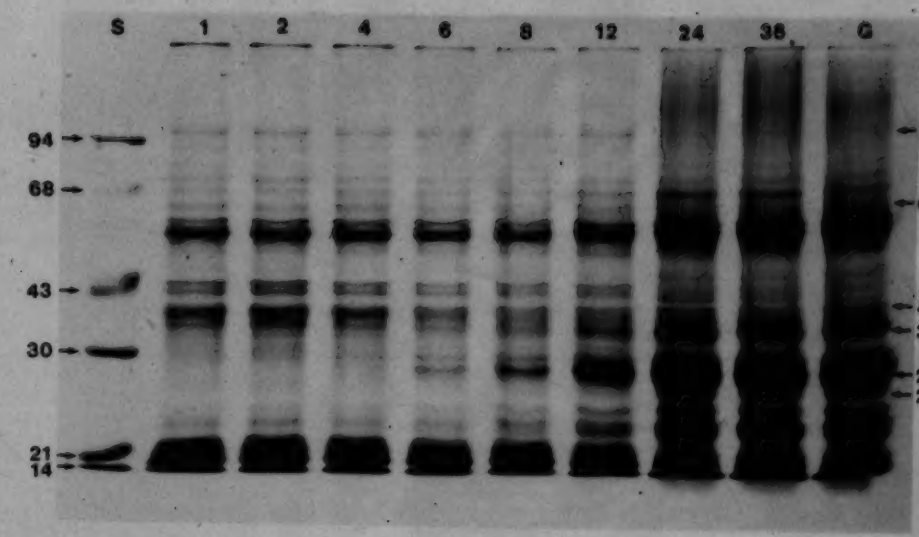

Fig. 12

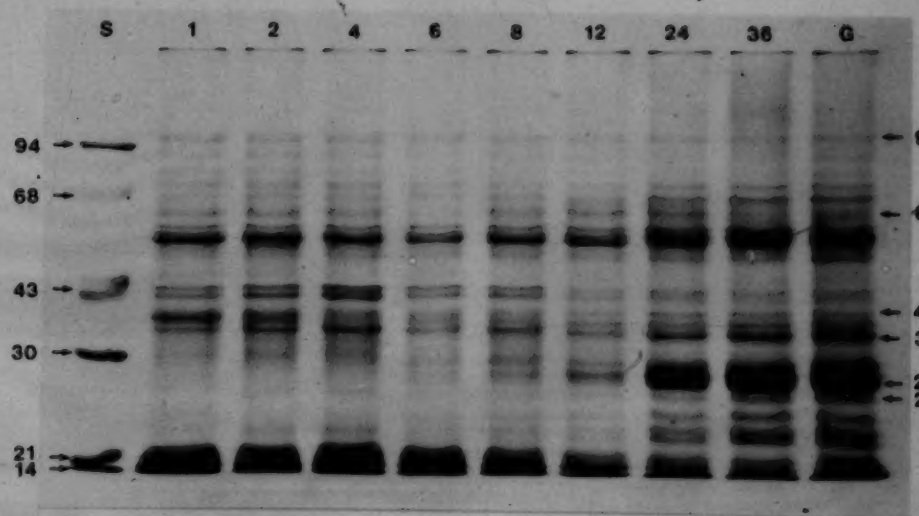

Fig. 11. SDS-PAGE of thylakoid membrane polypeptides from greening, normal barley seedlings etiolated for 6 days. Lane numbers represent the hours of greening following etiolation; S, molecular weight protein standards; and G, thylakoid membrane proteins from seedlings grown for 6 days under continuous illumination.

Fig. 12. SDS-PAGE of thylakoid membrane polypeptides from greening, mutant barley seedlings etiolated for 6 days. Lane numbers represent the hours of greening following etiolation; S, molecular weight protein standards; and G, thylakoid membrane proteins from seedlings grown for 6 days under continuous illumination.

bands between 22 Kd and 27 Kd also intensified during greening, however, most of these were also faintly visible after 2 hr.

Proteins extracted from plastids of greening M seedlings etiolated for 6 days are shown in Fig. 12. This pattern was virtually identical to that of the N plastids with only one exception; the time frame was delayed slightly. For example, significant amounts of the 29 Kd peptide seen in the N preparations after 8 hr of greening would be equivalent to the 12 hr M sample.

SDS-PAGE patterns of membrane proteins from seedlings etiolated for 8 days and then greened are shown in Fig. 13 (N) and Fig. 14 (M). The same patterns of appearance and disappearance of protein bands were obtained as in the 6 day samples, but the time frame has again been delayed. Although little change was seen in the time frame of the developmental sequence of the M patterns whether etiolated for 6 or 8 days, the 8 day N patterns have lagged considerably behind the 6-day N patterns to a developmental rate that was now almost indistinguishable from the M.

SDS-PAGE of the chlorophyll proteins is seen in Fig. 15 for the N and M seedlings grown for 6 and 8 days under continuous illumination. A sharp green band was seen at an apparent molecular weight of 96 Kd, which corresponds to the P700 chlorophyll a protein complex, a broader diffuse band is seen at 28 to 30 Kd which corresponds to the LHC, and a third band, at the gel front, was undoubtedly free pigment.

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Fig. 13

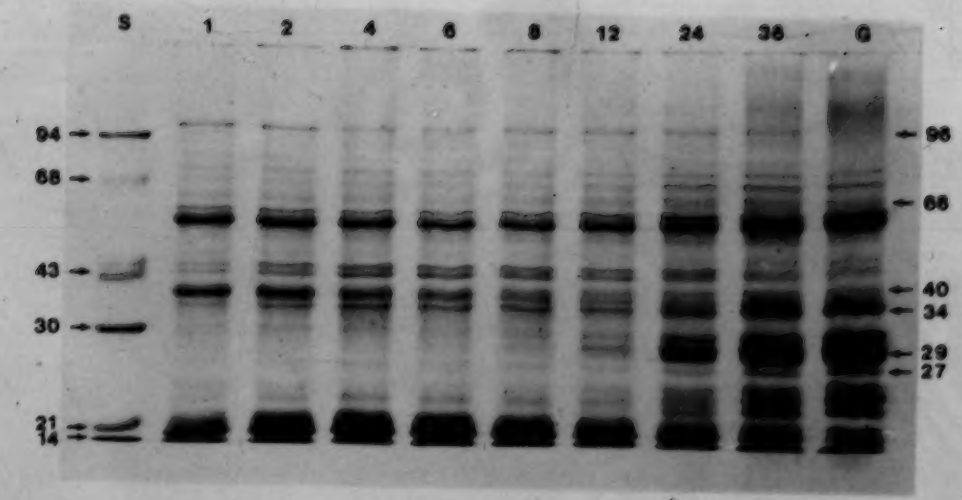

Fig. 14

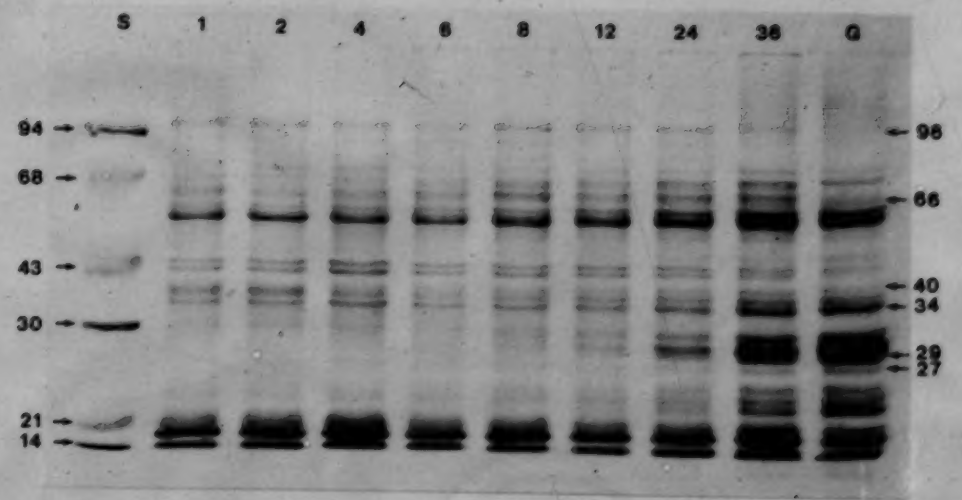

Fig. 13. SDS-PAGE of thylakoid membrane polypeptides from greening, normal barley seedlings etiolated for 8 days. Lane numbers represent the hours of greening following etiolation; S, molecular weight protein standards; and G, thylakoid membrane proteins from seedlings grown for 8 days under continuous illumination.

Fig. 14. SDS-PAGE of thylakoid membrane polypeptides from greening, mutant barley seedlings etiolated for 8 days. Lane numbers represent the hours of greening following etiolation; S, molecular weight protein standards; and G, thylakoid membrane proteins from seedlings grown for 8 days under continuous illumination.

Fig. 15

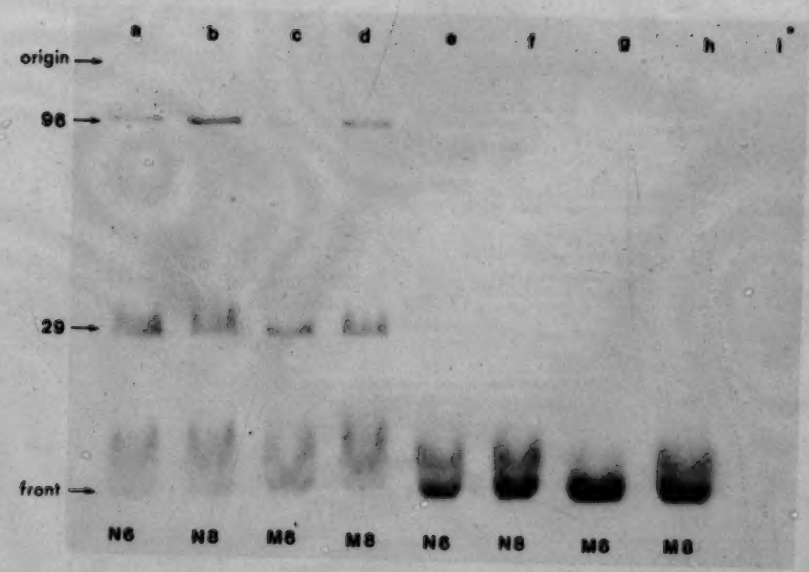

Fig. 16

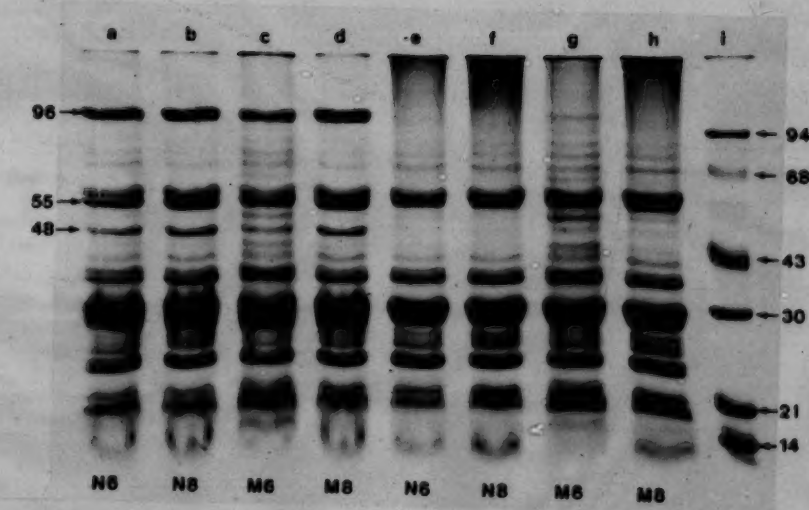

Fig. 15. Unstained gel after SDS-PAGE of chlorophyllins extracted from thylakoids of normal and mutant barley grown for 6 or 8 days in continuous light. lanes a-d, thylakoid membranes treated with SDS for 30 min at 5C; lanes e-h, membranes treated with SDS for 5 min at 100C; lane i, protein molecular weight standards.

Fig. 16. Stained gel after SDS-PAGE of chlorophyllins extracted from thylakoids of normal and mutant barley grown for 6 or 8 days in continuous light. Lane identification is the same as in Fig. 15.

These bands were visible and migrated to the same positions in the M as in the N preparations. When the membrane preparations were heated to 100C in SDS before PAGE, no chlorophyllin bands were visible in the unstained gels other than the free pigment band at the gel front.

For the electrophoresis of chlorophyll-proteins, the samples were not boiled with SDS before electrophoresis. When these unboiled samples were run beside boiled samples, however, the polypeptide patterns shown in Fig. 16 were produced. All the chlorophyll in the boiled samples ran with the dye marker. Boiling the samples also caused the disappearance of protein bands at 96 Kd, 55 Kd and 48 Kd, and a sharpening of the LHC band at 29 Kd. The unusual feature of these gels is that no band appeared in the boiled samples concomitant with the disappearance of the three bands. Thus, the proteins from the disappearing bands must have run to a position(s) which superimposed a band(s) already present in the samples, or must have failed to enter the gel.

4. Electronmicroscopic Studies

Chloroplasts of N and M leaf tissue from seedlings grown for 8 days under continuous illumination are shown in Fig. 17. Both types of chloroplasts have a highly developed internal membrane system. The M plastids however, are characterized by an irregular outline, a lack of organization in the alignment of the grana stacks, few stroma lamellae, and larger numbers of plastoglobules than

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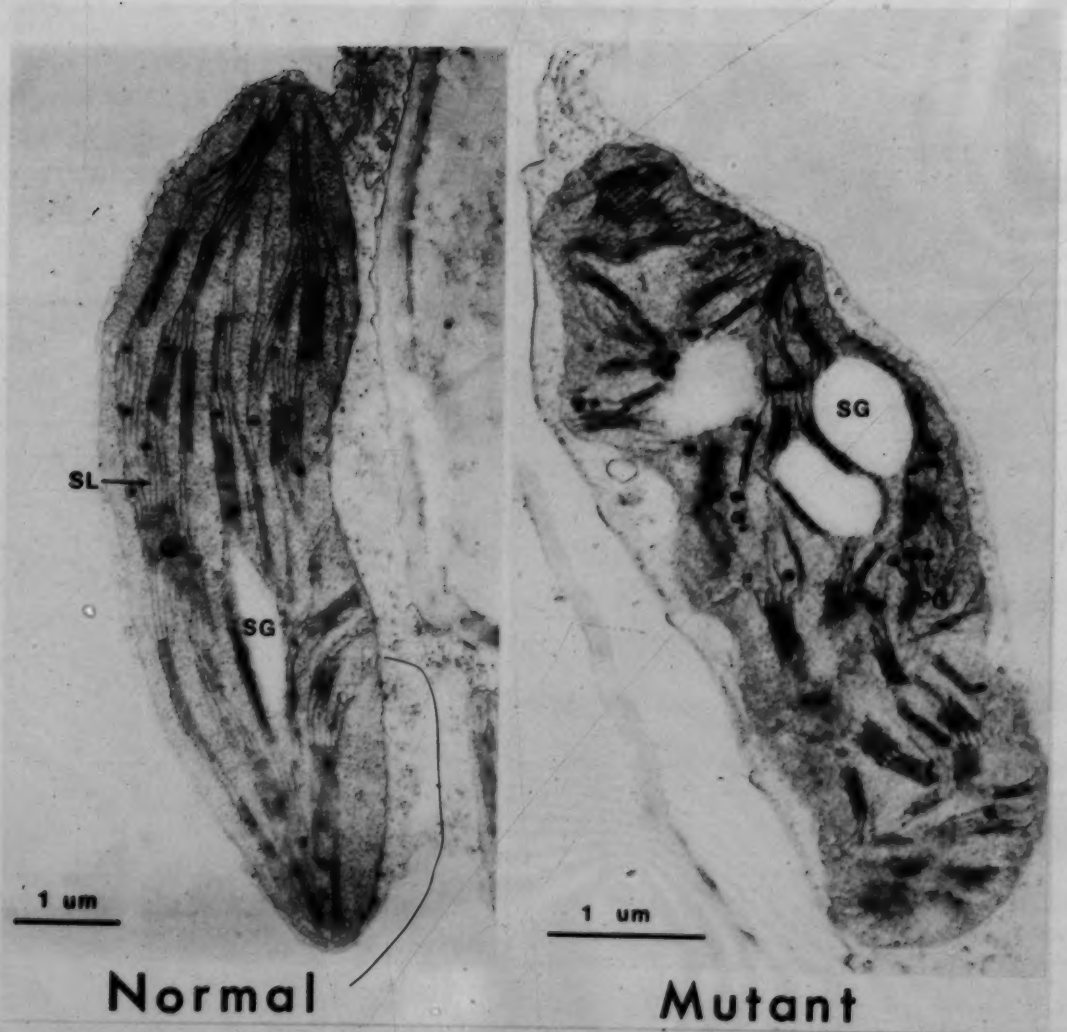

Fig. 17. TEM thin sections of chloroplasts from leaves of normal and mutant barley seedlings grown for 8 days under continuous light. G, grana; PG, plastoglobules; SG, starch grains; SL, stroma lamellae.

the N. These differences have also been previously noted (116).

It is difficult to compare organelle sizes from thin sections because the apparent size is dependent on the plane through which the organelle has been sectioned. The differences in size were more clearly represented by the scanning-electronmicrographs shown in Fig. 18. In order to make a direct comparison of N and M chloroplast sizes, a mixture of both types was also prepared for the SEM. In a side by side comparison, it was obvious that the M plastids were not only smaller, but they also appear to be more spherical than the N chloroplasts. Few broken chloroplasts and little contamination were seen in any of the SEM preparations.

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C. Discussion

Normal and M seedlings were kept etiolated for 6 or 8 days prior to greening in order to developmentally align the tissues to be compared . Even when kept in the dark for 6 days however, the subsequent greening rate was still slower in M compared to N seedlings (Fig. 3). After 8 days in the dark, followed by 24 hr in the light, very little difference was seen in the chlorophyll content of N and M leaves, whereas, when seedlings were grown in continuous light for 8 days, the N had substantially more chlorophyll than the M.

The rapid decline of the chlorophyll a/b ratio is a common indicator of chloroplast development following

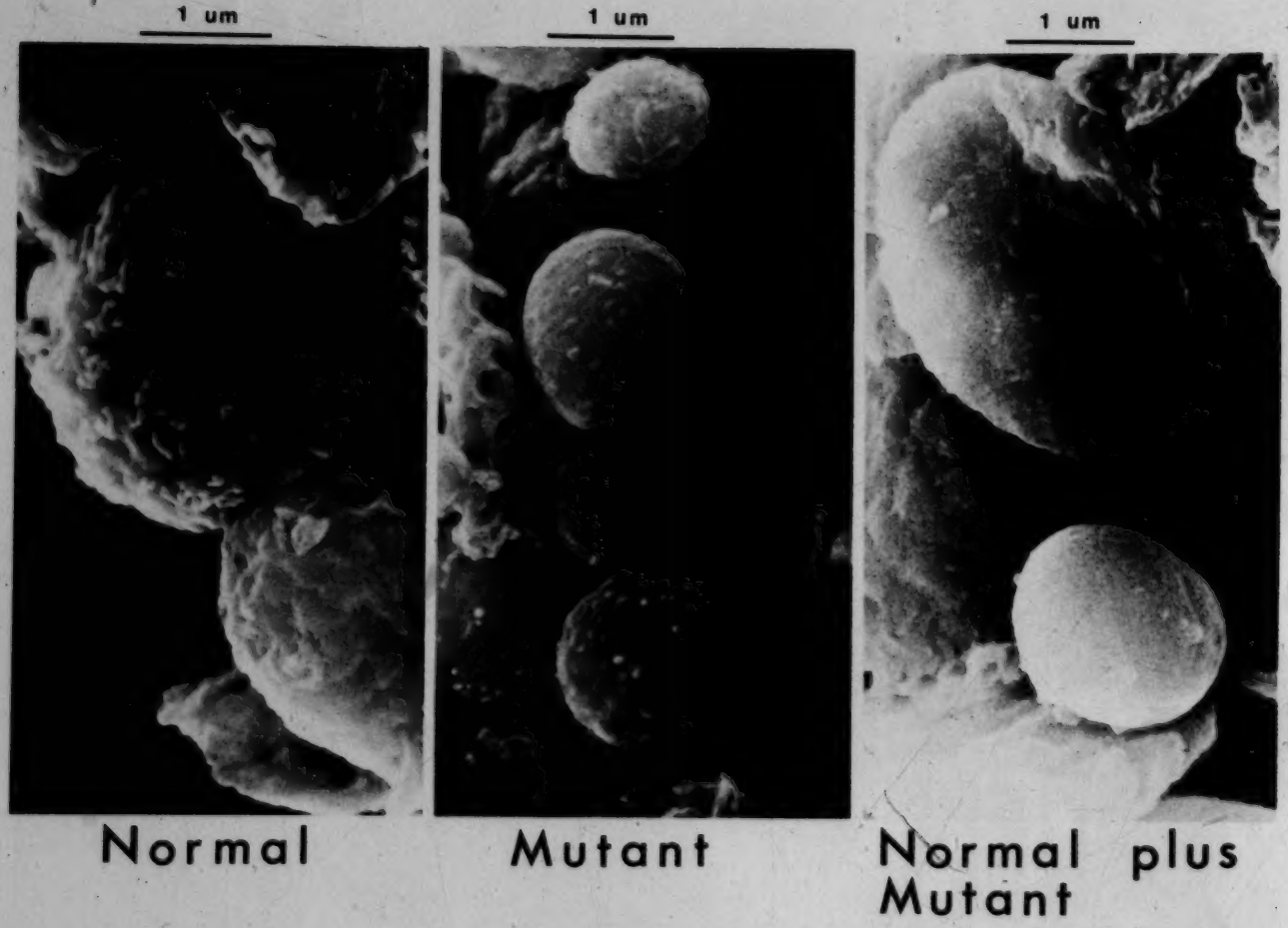

Fig. 18. SEM of isolated chloroplasts from normal and mutant barley seedlings grown for 6 days under continuous light.

etiolation (66, 98, 180, 240, 242). Other authors have reported that from 4 to 8 hr of illumination was required to achieve stable ratios in barley seedlings (66, 98). This greening rate however, was dependent on the environmental conditions (especially temperature and light intensity) during greening. In this study the time required for the chlorophyll a/b ratio to stabilize in the N was longer after 8 days of etiolation (Fig. 1 and 2) suggesting that a decrease in the rate of chloroplast membrane development after 8 days of etiolation had occurred. This was further substantiated by the reduced rates of chlorophyll synthesis in N seedlings after 8 days of etiolation compared with 6 days. The M, on the other hand, was not affected to any major extent by the longer duration in the dark as seen by little or no change in the rate of chlorophyll synthesis on a per plastid basis between 6 and 8 days (Fig. 4 and 5).

Chloroplasts increase substantially in volume upon greening, therefore, it was surprising that the protein content per plastid did not vary (Fig. 4 and 5). This suggests that the smaller etioplasts must contain a very high concentration of protein and that this concentration decreases during greening and plastid swelling. Incomplete breakage of the young etioplasts during the plastid membrane preparation however, could have resulted in erroneously high protein levels in these early stages. Prolamellar bodies are thought to contain a very high amount of protochlorophyll holochrome (124). It was not surprising that the M, which

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was deficient in prolamellar bodies (117), had a lower initial protein content per plastid than N etioplasts. The greened M however, had a higher protein content on a plastid basis than greened N chloroplasts. Since the protein content per plastid changed very little during chloroplast development, a catabolism of membrane proteins may have occurred producing substrates for the formation of new proteins. Alternatively, the insertion of cytoplasmically synthesized proteins along with the loss of certain etioplast proteins would also result in little change in the protein content per plastid during development. Good candidates for these storage proteins might be the high molecular weight proteins seen in Figures 11 to 14, which disappear as a function of greening. The breakdown of these proteins might be responsible for the strongly staining bands of very low molecular weight seen during the first few hours of greening.

Analysis of the polypeptide profiles in Fig. 10 indicated that no significant thylakoid membrane protein present in the N was absent from the M chloroplasts. Jhamb and Zalik (118) also found no difference in gel patterns after the 8 day stage, but differences were noted at the 6 day stage. This disagreement in results could be reconciled by differences in chloroplast preparation or the methodology of electrophoresis.

Several of the polypeptide bands could be identified by homology of the apparent molecular weights with those of

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known proteins. For example, the alpha and beta subunits of the coupling factor have molecular weights of 59 Kd and 56 Kd respectively (167), the LHC apoprotein was 27-29 Kd (126, 241), and the P700 chlorophyll a protein complex was known to be about 96 Kd (244). Bogorad and coworkers have used a 32 Kd photoinduced polypeptide for the study of chloroplast genetics (86). In Figures 11 -14 a major photoinduced polypeptide at 34 Kd can be seen, which probably corresponds to the photoinduced 32 Kd protein found by others (86, 192).

The developmentally related appearance and disappearance of chloroplast membrane proteins in barley was also recently studied by Hoyer-Hansen and Simpson (106). These authors stated that although 15 polypeptides disappeared during greening, changes in the polypeptide patterns from 3-24 hr were primarily quantitative. The molecular weights were not identified but their data also indicated a general pattern of loss of high and low molecular weight proteins as a function of greening.

Chlorophyll b is an integral component of the LHC and the appearance of the LHC band coincided with the stabilization of the chlorophyll a/b ratio. After 6 days of etiolation, the appearance of the LHC and the stabilization of the chlorophyll a/b ratio occurred earlier in the N than the M chloroplasts. After 8 days of etiolation, the chlorophyll a/b ratio took longer to stabilize and the LHC was not visible in the gels until correspondingly longer greening times. Thus, as in the case of chlorophyll

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accumulation, changes in the chlorophyll a/b ratio, and changes in the polypeptide profiles there was a developmental time lag in the M which was not corrected by prolonged etiolation. Rather, the M approached N parameters because of a decrease in the developmental rate of the N after prolonged etiolation. In other words, extended etiolation had a detrimental effect on the N seedlings but little or no effect on the M seedlings.

The chlorophyll-protein patterns in Fig. 15 showed only 3 green bands immediately after electrophoresis. These correspond to the P700 chlorophyll a protein complex (96 Kd), LHC (29 Kd) and a zone of free pigment at the gel front. These gels were from seedlings greened under continuous illumination for 6 and 8 days and the patterns for the N and M were the same at both stages. The 6 day M had less chlorophyll in the chlorophyll-protein bands and free pigment zone, as equivalent amounts of protein, not chlorophyll, were loaded on the gels. The chlorophyll was completely lost from the chlorophyll-protein bands if the samples were boiled in SDS before application (Fig. 15). Another consequence of this procedure was the loss of protein bands at 96 Kd, 55 Kd and 48 Kd. A loss of protein bands at 95 Kd, 53 Kd and 47 Kd were reported by Wessels and Borchert (241) upon boiling spinach chloroplast membranes in SDS for 2-5 min, or delipid acetone before SDS-PAGE. They further suggested that the 47 Kd band is the PSII reaction centre (see also 51), and the 53 Kd band is a dimer of the

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LHC. Although no chlorophyll was detected in these areas immediately after electrophoresis (Fig. 15), bands at 55 Kd and 48 Kd (Fig. 16) were lost upon boiling in SDS as were those previously reported (241). Estimates of the molecular weight for the LHC vary from 21-29 Kd (226). Before boiling the LHC band was broad and ranged from 25-30 Kd. After boiling in SDS however, the bands sharpened and a dimer could be distinguished around 29 Kd in all samples. Thus, we have evidence for the presence of the LHC, PSII reaction centre, and the P700 chlorophyll a protein in both the N and M seedlings after 6 and 8 days of continuous illumination.

The plastoquinone A analysis generated an unexpected result. More plastoquinone per unit chlorophyll was present in the M than the N after both 6 and 8 days of continuous illumination (Table I). Assuming the photosynthetic unit size was unaffected by the mutation (an assumption supported later in Section IV of this thesis), and a photosynthetic unit size of about 400 chlorophylls/photosynthetic unit (112), then N seedlings greened for 8 days would have about 10 plastoquinones/unit and the M about 16 per unit. These values were slightly lower than those previously reported (19,220). It must be realized however, that this plastoquinone may not all be used in PET. Indeed, plastoglobules or osmophilic granules seen by EM have been shown to contain quinones (145) and larger numbers of plastoglobules have been reported in the M chloroplasts than in the N chloroplasts (117).

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The M seedlings grown under continuous illumination exhibited consistently lower chlorophyll a/b ratios than the N. Low chlorophyll a/b ratios have been associated with shade-type or grana-rich chloroplasts (25). Although the M may not be considered grana-rich, it could be considered stroma lamellae-poor (Fig. 17). The reduced amount of stroma lamellae may give rise to the disorder of the grana seen in the mutant. It also has been suggested previously that a lack of chloroplast microtubules might be the cause of the improper grana orientation (116).

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IV. FUNCTIONAL STUDIES

A. Materials and Methods

1. Plant Material and Chloroplast Isolation

Plant growth conditions, harvesting and preparation of chloroplasts were carried out as previously described for the structural studies. The methods were the same in order to facilitate comparisons between structural and functional changes.

2. Chemicals

FeCN, DL-glyceraldehyde, DPIP, TMPD, and MV were all purchased from Sigma. DMQ and DPC were obtained from Eastman Kodak Co., Rochester, N.Y. The DBMIB was a gift from Dr. E. Tyszkiewicz.

3. Photosystem I and Photosystem II Assays

a) H2O ---> FeCN. This measurement of whole photosystem electron transport was carried out using a Hansatech oxygen electrode connected to a Fisher Recordall 5000 chart recorder. The O2 evolution accompanying H2O splitting with FeCN as an electron acceptor has been described by Delieu and Walker (61). The sample cell had a total volume of 1.0 ml and contained the chloroplast preparation (5-20 ug of chlorophyll), 1.5 mM FeCN, and 10 mM DL-glyceraldehyde. The sample was stirred in the dark at 20C until the recorder stabilized (about 2 min) and then illuminated with a 500 w projector lamp fitted with a Corning 2-62 red glass filter. The light intensity at the cell was approximately 950

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Kerg/cm²/sec as determined with a pyrheliometer (Eppley model 10). After 30 sec of illumination, 20 ul of 0.5 M NH4Cl was added to the cell to obtain a rate for the uncoupled reaction. The O2-electrode was calibrated by making a full scale recorder deflection equivalent to the oxygen concentration of air saturated water. The maximum value thus corresponded to 280 uMO2 (61) and a zero O2 value was obtained after adding a few crystals of sodium dithionite.

b) H2O ---> DPIP. PET using DPIP as the electron acceptor was carried out spectrophotometrically with a Varian Techtron dual beam spectrophotometer by a method similar to that of Woo et al.(247). Again, illumination was provided by a 500 W projector lamp but light was filtered through a Corning 2-62 red glass filter, a 10 cm water/heat filter and a 500 ml round bottom flask filled with water acted as a condensing lens. To a 3.0 ml quartz cuvette were added in sequence: 1.85 ml of the chloroplast preparation (1-2 ug chlorophyll/ml); 1.0 ml of 30 mM phosphate buffer (pH 7.4 at 22C)(Na2HPO4/KH2PO4) containing 1.0 M sorbitol and 3 mM MgCl2; and finally 150 ul of 2 mM DPIP added slowly as a layer on the surface. The contents of the cuvette were then mixed by inversion, immediately placed in the spectrophotometer and adjusted to 0.500 O.D. at 595 nm against a similarly prepared sample without chloroplasts. The cuvette was then placed in the saturating light beam for 15 sec intervals, recording the absorbance at 595 nm after

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each successive illumination. The changes in absorbance were plotted and the slopes determined. Using the mM extinction coefficient of 17.4 for DPIP (247) the activity of the sample was calculated from the following formula:

$$\frac{\text{slope (O.D. units/hr) x 1000}}{\text{chlorophyll concentration (mg/ml) X 17.4}}$$

= uM DPIP reduced / mg chl / hr

These values were corrected by subtraction of the DCMU-insensitive reduction of DPIP. This was determined by repeating the experiment with the addition of 10 ul of 300 uM DCMU (1uM final concentration) in MeOH.

c) DPC ---> DPIP. The photoreduction of DPIP using DPC as an electron donor was carried out essentially as described above for the H2O ---> DPIP assay. However, immediately after layering the DPIP solution on the sample in the cuvette, 15 ul of 100 mM DPC (0.5 mM final concentration) made up in MeOH, was added. The contents of the cuvette were mixed by inversion and the subsequent procedures were carried out as described above. Again, the values were corrected for DCMU-insensitive reduction of DPIP.

4. Photosystem I Assay. TMPD --> MV

Photosystem I was assayed polarographically using the Hansatech oxygen electrode. The procedure was identical to

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that for H2O --> FeCN except the reaction mixture contained chloroplast membranes (1-2 ug chlorophyll/ml, 4 mM TMPD, 2 mM methyl viologen, 60 mM sodium ascorbate and 1 mM DCMU. DCMU blocks the PSII-catalysed O2 evolution, and upon illumination, O2 uptake is measured. The O2 is consumed in a Mehler-type reaction of reduced MV.

5. Photosystem II Assay. DPC --> DMQ

This Photosystem II specific photoreduction was measured polarographically with the Hansatech oxygen electrode as in the TMPD-->MV assay. The reaction mixture contained the chloroplast membranes (10-20 ug chlorophyll/ml), 0.5 mM DPC, and 2 mM DMQ (115). After 20-30 sec of O2 evolution, 20 ul of 0.5 M NH4Cl was added to obtain an uncoupled rate. All experiments were repeated with the addition of 10 ul of 1 uM DBMIB in MeOH, to inhibit any PET beyond plastoquinone.

6. Light Saturation Measurements

The light saturation experiments were carried out using the DPC --> DPIP assay. The light intensity of the projector was varied with neutral density filters (Balzers) so that the light intensity could be changed without affecting the light quality. For measurement of activity using filters with less than 10% transmittance, the experiments were done in semi-darkness to reduce any contribution of stray light. All samples were corrected for nonphotosynthetic reduction of DPIP by subtracting the activity of a sample kept in total darkness.

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7. DCMU - Saturation Measurements

The DCMU saturation experiments were also carried out using the DPC --> DPIP assay system. The experiments were carried out in saturating light (1,100 Kerg/cm²/sec), and the DMCU concentration was varied from 0.01 uM to 10 uM. As the DCMU was made up in MeOH, the dilutions were made prior to addition to the sample so that a constant volume of MeOH was added in each experiment. The activity at 10 uM DCMU was used as a correction factor for nonphotosynthetic reduction of DPIP.

B. Results

1. Photosystem II and I

Total PET was measured as oxygen evolution by membrane preparations upon illumination using FeCN as the electron acceptor. The development of this photochemical activity on a unit chlorophyll basis in greening etiolated seedlings is shown in Figures 19 and 20. No O2 evolving activity could be detected within the first 4 hours for either N or M membranes following 6 days of etiolation. The rate of development of the PET capacity was faster in the N than in the M, and this higher rate was not affected by the duration of the dark pretreatment. All plastid preparations had achieved activities of nearly 100% that of the light grown controls within 24 hr of greening, although consistently higher activities were observed in N plastid preparations. Since the level of chlorophyll was reduced in the M, it was

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Fig. 19

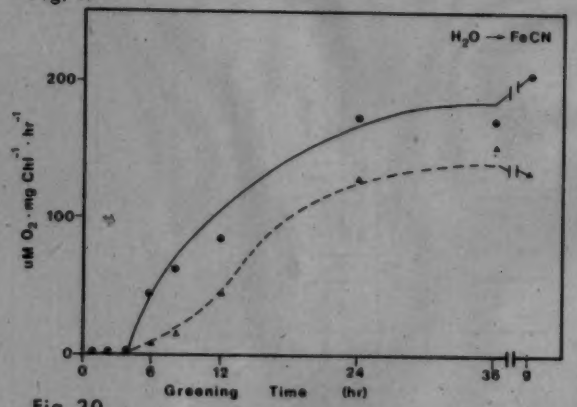

Fig. 20

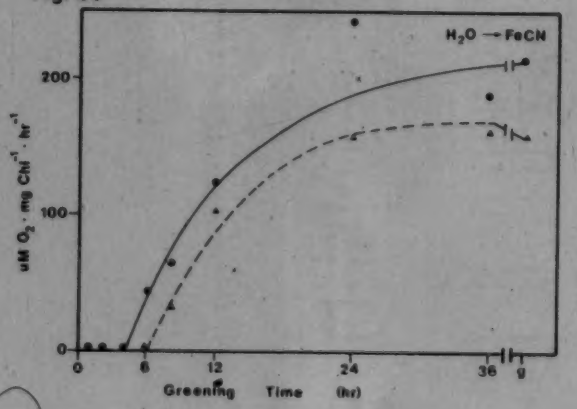

Fig. 19. Development of H2O-->FeCN activity on a chlorophyll basis in chloroplasts from normal and mutant seedlings etiolated for 6 days. (●), normal; (▲), mutant; g, values for seedlings grown for 8 days under continuous illumination. The values plotted are the means of four replicates.

Fig. 20. Development of H2O-->FeCN activity on a chlorophyll basis in chloroplasts from normal and mutant seedlings etiolated for 8 days. (●), normal; (▲), mutant; g, values for seedlings grown for 8 days under continuous illumination. The values plotted are the means of four replicates.

Fig. 21

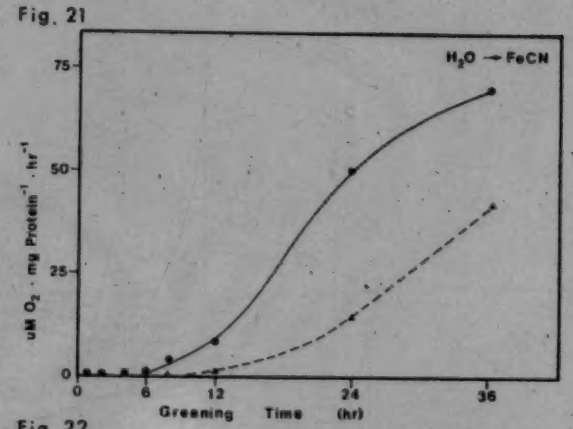

Fig. 22

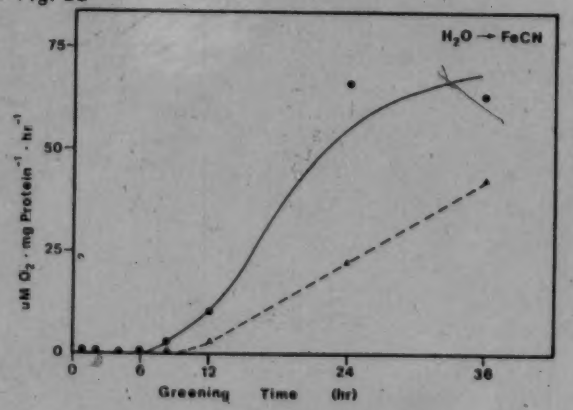

Fig. 21. Development of H2O-->FeCN activity on a protein basis in chloroplasts from normal and mutant seedlings etiolated for 6 days. (●), normal; (▲), mutant. The values entered are the means of four replicates.

Fig. 22. Development of H2O-->FeCN activity on a protein basis in chloroplasts from normal and mutant seedlings etiolated for 8 days. (●), normal; (▲), mutant. The values plotted are the means of four replicates.

of value to interpret the data on a unit protein basis (Fig. 21 and 22). As seen from these curves the development of M membrane activities was far from complete at 24 hr, and preparations from N chloroplasts were the first to reach a stable activity level. An increase in activity on a protein basis with no change on a chlorophyll basis can only be explained by either a drop in plastid protein between 24 and 36 hr, or an increase in chlorophyll associated with the increase in activity. Clearly the former cannot be supported by the data in Fig. 4 and 5. Thus, the increase in rate of PET between 24 and 36 hr of greening in the M plastids is directly related to an increase in chlorophyll content.

Whole chain PET was also measured spectrophotometrically by the photoreduction of DPIP with water as electron donor (Fig. 23 and 24). The photoreduction of DPIP was very high in the presence of DCMU during the first few hours of greening therefore all measured activities had to be corrected for DCMU-insensitive photoreduction in order to get a valid measure of the electron flow through PSII. Photochemical activity on a chlorophyll basis showed a rapid rise to a maximum after 3-4 hr of greening in both N and M thylakoids after 6 days of etiolation followed by a decline to a stable level at the 12 hr stage. The activities peaked after 6 and 9 hr for N and M plastids respectively after 8 days of etiolation and stable activities were not obtained until at least 24 hr of greening. The H2O --> DPIP activities in light grown

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Fig. 23

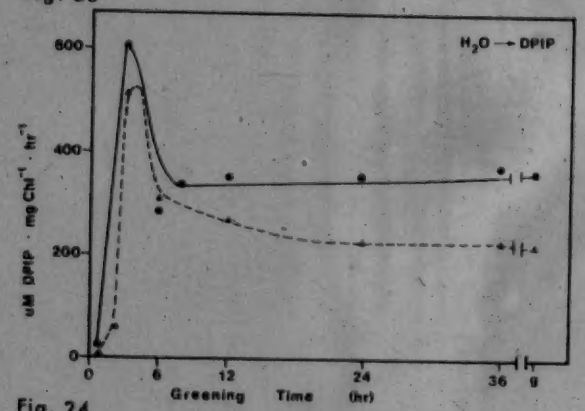

Fig. 24

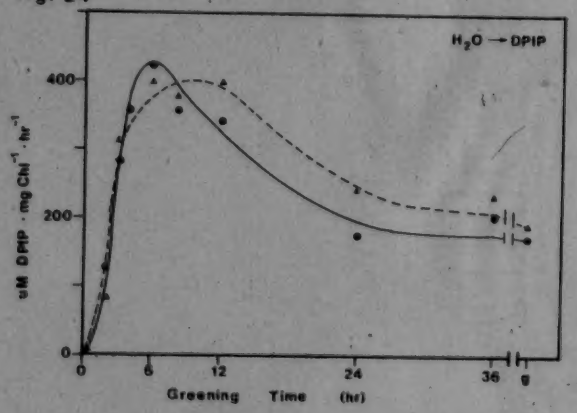

Fig. 23. Development of H2O-->DPIP activity on a chlorophyll basis in chloroplasts from normal and mutant seedlings etiolated for 6 days. (●), normal; (▲), mutant; g, values for seedlings grown for 6 days under continuous illumination. The values plotted are the means of four replicates.

Fig. 24. Development of H2O-->DPIP activity on a chlorophyll basis in chloroplasts from normal and mutant seedlings etiolated for 8 days. (●), normal; (▲), mutant; g, values for seedlings grown for 8 days under continuous illumination. The values plotted are the means of four replicates.

Fig. 25

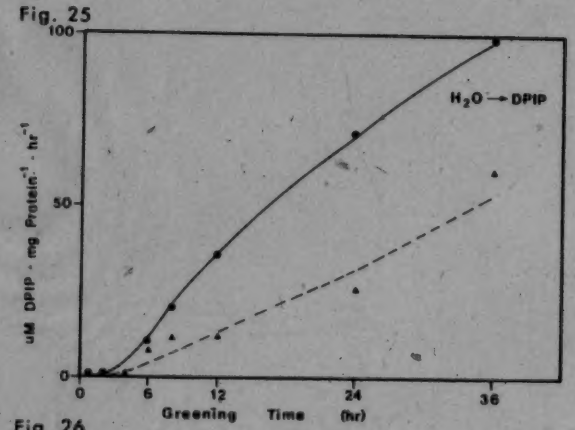

Fig. 26

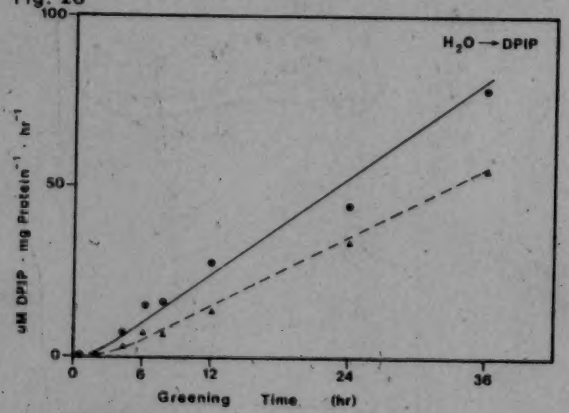

Fig. 25. Development of H2O-->DPIP activity on a protein basis in chloroplasts from normal and mutant seedlings etiolated for 6 days. (●), normal; (▲), mutant. The values plotted are the means of four replicates.

Fig. 26. Development of H2O-->DPIP activity on a protein basis in chloroplasts from normal and mutant seedlings etiolated for 8 days. (●), normal; (▲), mutant. The values plotted are the means of four replicates.

seedlings were higher for the N than the M after 6 days, but almost equal after 8 days of continuous illumination. High activities per unit chlorophyll during early stages of greening are easily misinterpreted since the actual amounts of chlorophyll in the plastid are very small. When the data are plotted as activity per unit protein (Fig. 25 and 26), a continuous increase in activity is seen over the 36 hr of development. The rate of increase in activity of the M chloroplasts is unaffected by the duration of the dark pretreatment but that of the N is considerably reduced after 8 days in the dark. In all cases the activity per unit protein continues to rise long after stable rates are reached on a chlorophyll basis. Thus, as in the H2O --> FeCN data, the increase in chlorophyll during the later stages of greening in the M plastids contributes directly to the increase in measured activity.

The artificial electron donor, DPC, was used to circumvent the Hill reaction. The additional electron transport stimulated by DPC over that with H2O as the donor is shown in Figures 27 and 28. Again, the activities were corrected for DCMU-insensitive photoreduction to negate the effects of any DPC donation to PS I. During the first few hours of greening, DPC is a potent donor to PSII. This stimulation declines sharply over the first 4 hr in seedlings etiolated for 6 days as the H2O-->DPIP activity becomes functional. This sharp decay component is extended to 8 and 12 hr for the N and M respectively in plants

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Fig. 27

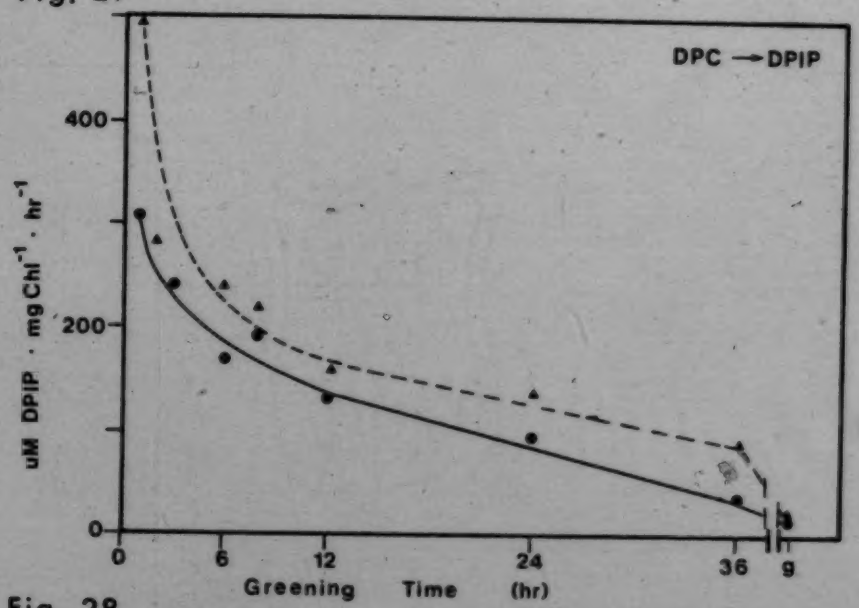

Fig. 28

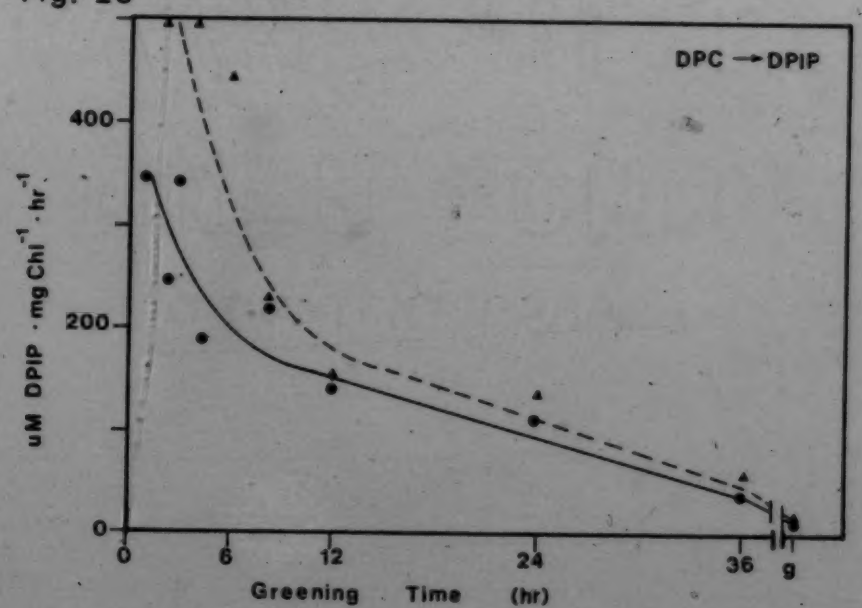

Fig. 27. Photoreduction of DPIP stimulated by DPC as a function of greening in chloroplasts from normal and mutant seedlings etiolated for 6 days. (●), normal; (▲), mutant. The values plotted are the means of four replicates.

Fig. 28. Photoreduction of DPIP stimulated by DPC as a function of greening in chloroplasts from normal and mutant seedlings etiolated for 8 days. (●), normal; (▲), mutant. The values plotted are the means of four replicates.

greened after 8 days of etiolation. The rate of decrease over the 12-36 hr greening period is fairly uniform for both N and M after both 6 and 8 days of etiolation.

2. Photosystem I

The development of photochemical activity associated with Photosystem I is shown in Figures 29 and 30. Significant levels of activity were obtained in all cases within the first hour of greening. Maximum rates of PSI activity, on a chlorophyll basis, were seen after 8 hours of greening in both N, and M plants etiolated for 6 days, whereas, after 8 days, maximum activities were reached after only 4 hours of greening. PSI activity of N thylakoid membranes was higher during the initial stages of greening but attained a stable rate after 24 hr which was lower than that of the M. After 8 days of etiolation, although the maximum activities were achieved earlier in plastid development, the photochemical activities had not reached stable levels even after 36 hr of development.

As previously mentioned, the initial activities were artificially high due to the low chlorophyll content of the plastids in the early stages of greening. When plotted on a protein basis (Figures 31 and 32), the rates of PSI development were consistently higher in the N regardless of the duration of etiolation. Except for the M after 6 days of etiolation, all PSI activities began to level off after about 24 hr of greening. On a unit protein basis, very little activity was apparent in the first hour of greening,

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Fig. 29

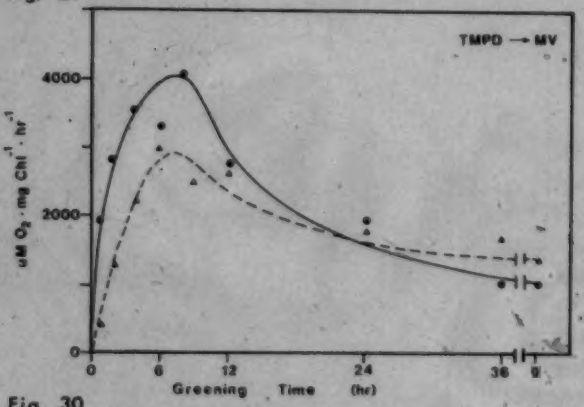

Fig. 30

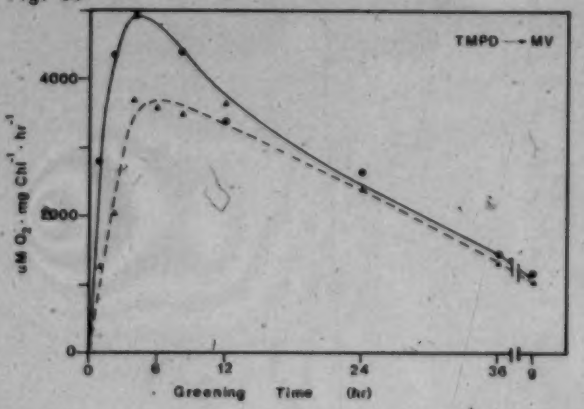

Fig. 29. Development of TMPD-->MV activity on a chlorophyll basis in chloroplasts from normal and mutant seedlings etiolated for 6 days. (●), normal; (▲), mutant; g, values for seedlings grown for 6 days under continuous illumination. The values plotted are the means of four replicates.

Fig. 30. Development of TMPD-->MV activity on a chlorophyll basis in chloroplasts from normal and mutant seedlings etiolated for 8 days. (●), normal; (▲), mutant; g, values for seedlings grown for 8 days under continuous illumination. The values plotted are the means of four replicates.

Fig. 31

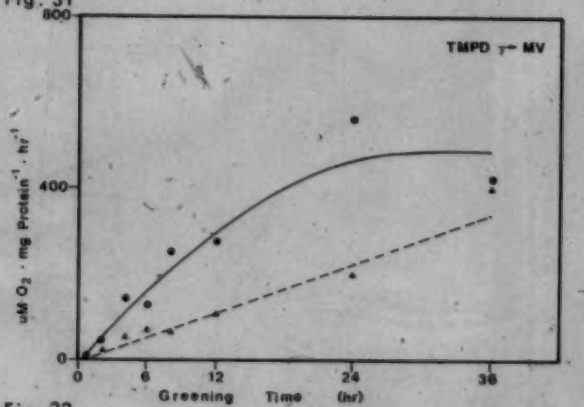

Fig. 32

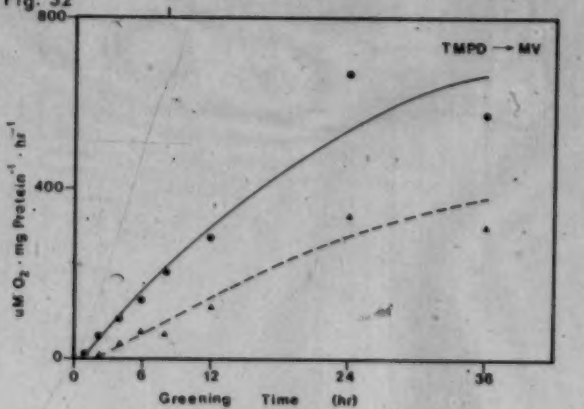

Fig. 31. Development of TMPD-->MV activity on a protein basis in chloroplasts from normal and mutant seedlings etiolated for 6 days. (●), normal; (▲), mutant. The values plotted are the means of four replicates.

Fig. 32. Development of TMPD-->MV activity on a protein basis in chloroplasts from normal and mutant seedlings etiolated for 8 days. (●), normal; (▲), mutant. The values plotted are the means of four replicates.

however, significant activity was seen after 2 hr. Thus, PSI appeared earlier and reached stable levels sooner during the greening process than PSII.

3. Photosystem II

Photosystem II activity using the DPC --> DMQ assay was measured for N and M seedlings exposed to continuous illumination for 6 and 8 days. These data are presented in Table II. The activity in the presence of the inhibitor DBMIB is an estimate of the electron flow through PSII and, after 6 days of illumination, plastids from N seedlings exhibited higher activities than those of the M. Between 6 and 8 days the M activity in the presence of DBMIB changed very little but the activity of the N plastids dropped by 37%. As a result, after 8 days there was no difference in PSII activities between N and M chloroplasts.

In the presence of DBMIB, the uncoupler NH_4Cl, had no effect on the PSII rate. This was not unexpected since DPC supplies electrons on the reducing side of the first coupling site (H_2O oxidation), and DMQ accepts electrons on the oxidizing side of the second coupling site (plastoquinone). Thus no proton gradient would be set up, and an uncoupler such as NH_4Cl should have no effect. In the absence of the uncoupler, the inhibitor DBMIB had no effect on the measured PSII activity. Thus, all the electrons were reducing DMQ at the maximum possible rate and were not being lost to PSI reactions. When the uncoupler was added in the absence of the inhibitor though, the reduction of DMQ was

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Table II. Photochemical activity of the Photosystem II reaction center (DPC-->DMQ) in chloroplasts from N and M seedlings grown for 6 or 8 days under continuous light.

Sample	PS II Activity*	
	Normal	Mutant
6 days		
DMQ	121	97
DMQ + NH4Cl	193	160
DMQ + DBMIB	119	87
DMQ + NH4Cl + DBMIB	130	87
8 days		
DMQ	82	108
DMQ + NH4Cl	156	173
DMQ + DBMIB	85	92
DMQ + NH4Cl + DBMIB	94	106

* values given are uMO2/mg Chl/hr

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stimulated by over 60%.

4. Light Saturation Measurements

In order to make comparisons of the photosynthetic unit sizes of N and M chloroplasts, the effects of changes in the light intensity on the PET were measured by the DPC --> DPIP reaction. After 6 days of continuous illumination the maximum photochemical activity (Vmax) of the N was higher than that of the M. However, the light intensity at which the activity was saturated was the same for both N and M chloroplasts (Fig. 33). This is clearly shown in the double reciprocal plots of the data (Fig. 33 insert), where extrapolations of these lines converge on the ordinate. Thus, both N and M had the same half-saturation point (Km). This was also the case for the seedlings grown for 8 days under continuous illumination (Fig. 34). Identity of the light saturation points can be related to equal numbers of chlorophyll per trap, or, in other words, equal photosynthetic unit sizes. Identical light saturation points with a lower maximum reaction velocity however, can only be interpreted as a lower efficiency of the PET in the M. The lower efficiency of the PET in the M is clearly demonstrated in the light limiting regions of the curves. It has been suggested that the slope of the line in the region of the light saturation curve where light is limiting is proportional to the quantum yield (97)

The development of the photosynthetic units as defined by light saturation kinetics is shown in figures 35 and 36

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Fig. 33

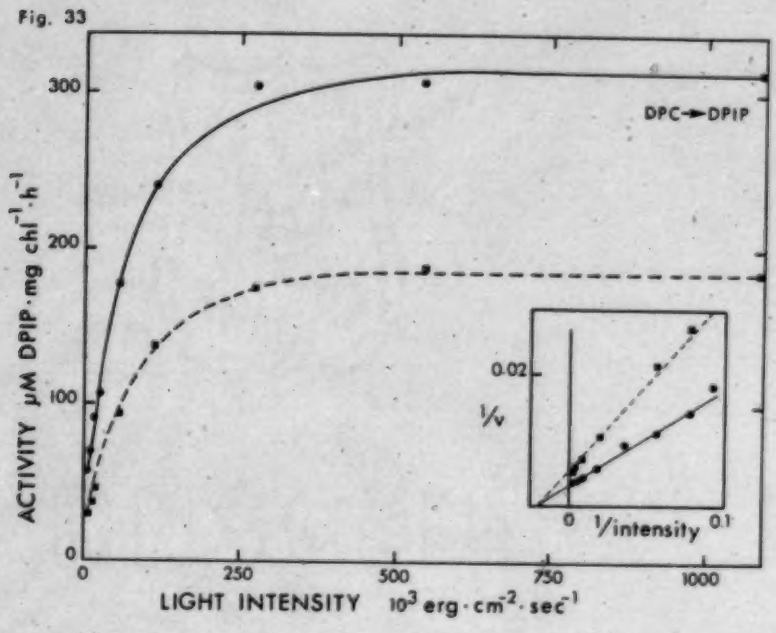

Fig. 34

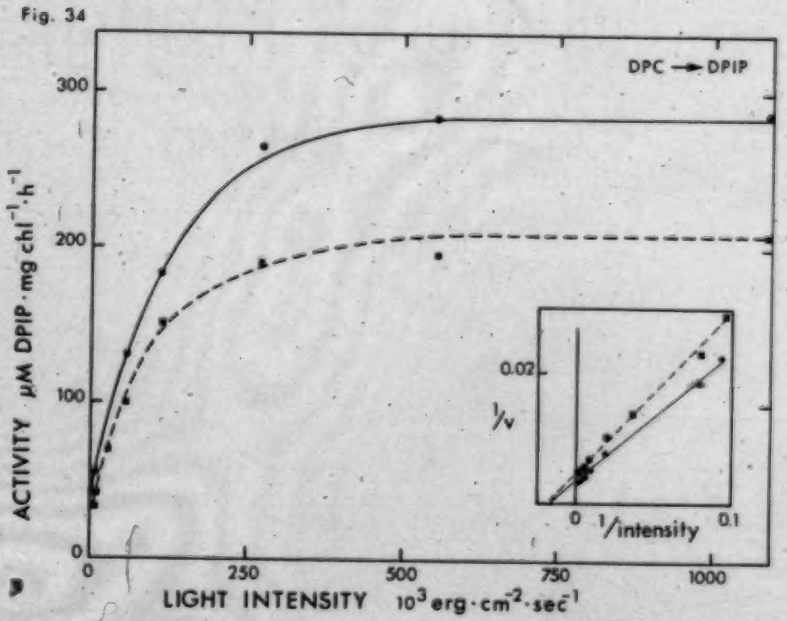

Fig. 33. Light saturation curves for chloroplasts from 8 day normal and mutant seedlings. Photoreductive activities of the DPC-->DPIP reaction are given for normal (●) and mutant (■) chloroplasts. The double reciprocal plots of these data are shown in the insert. The values plotted are the means of four replicates.

Fig. 34. Light saturation curves for chloroplasts from 8 day normal and mutant seedlings. Photoreductive activities of the DPC-->DPIP reaction are given for normal (●) and mutant (■) chloroplasts. The double reciprocal plots of these data are shown in the insert. The values plotted are the means of four replicates.

for 6 day etiolated N and M seedlings respectively. These data have been normalized so that the activity at the maximum light intensity is equal to one. It is apparent from these curves that the light intensity required for saturation of the photochemical activity decreased as greening progressed. The Vmax also changed during greening. Nevertheless, the light-limiting regions of the curves indicated an increase in efficiency of the photochemical activity during development. The double reciprocal plots shown in Figures 35 and 36 indicate that the half saturation intensities decreased from 210 to 69 Kerg/cm²/sec during the first 24 hr of greening in the N, while it decreased from 470 to 66 Kerg/cm²/sec over the same time period in M chloroplasts. Based on these results, the photosynthetic unit size increased more rapidly in the N, achieving light saturation points approximating those of light-grown controls after only 12 hrs of greening. The M, on the other hand, took about 24 hrs to attain the light grown control rates.

Similar data were obtained for plants etiolated for 8 days before greening (Fig. 37 and 38). However, the rate of increase in the photosynthetic unit size was less in these 8 day etiolated N seedings, since light saturation points equal to the light grown controls were not realized until the 24 hr stage. In the M, the rate of increase in the photosynthetic unit size was unaffected by the duration of dark pretreatment. The half-saturation intensities decreased

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Fig. 35

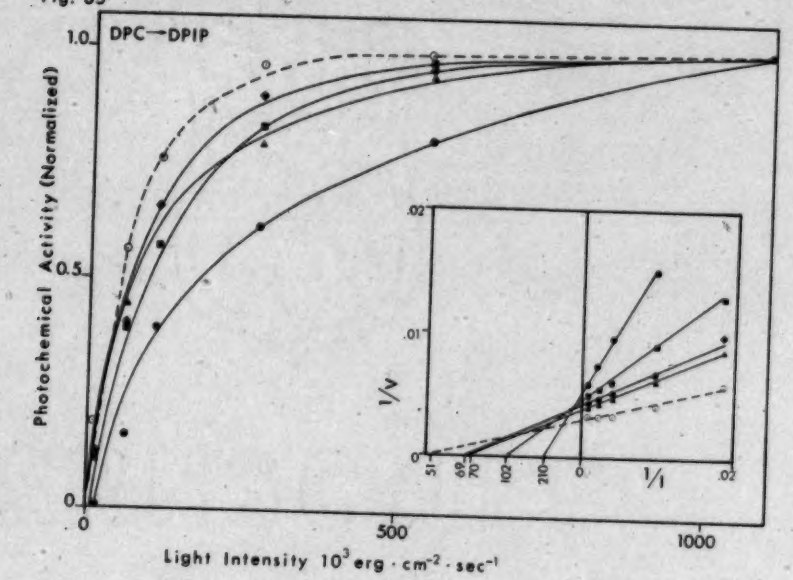

Fig. 36

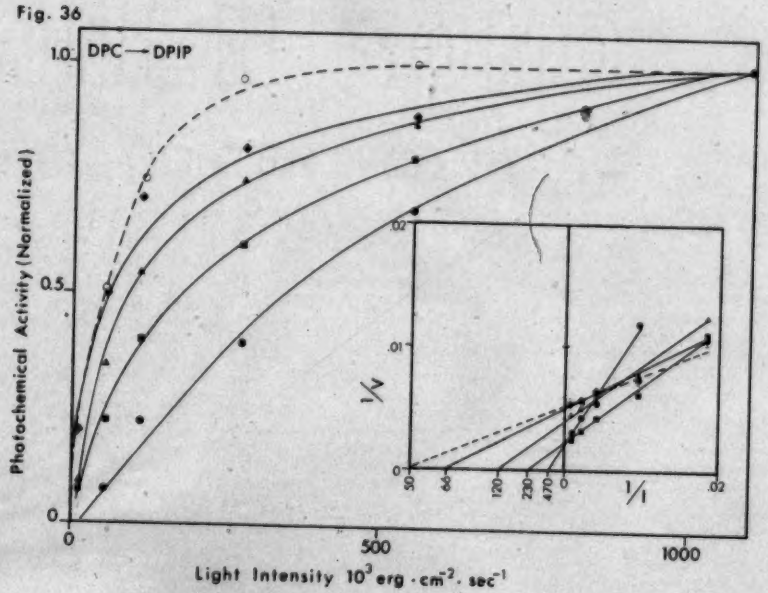

Fig. 35. Light saturation curves for developing normal chloroplasts from seedlings etiolated for 6 days. The photoreductive activities have been normalized to a value of 1.0 at the maximum light intensity. Data are shown for chloroplasts greened for 2 hr (●), 6 hr (■), 12 hr (▲) and 24 hr (◆) as well as for chloroplasts from seedlings grown for 6 days under continuous illumination (○). The double reciprocal plots of the actual data are shown in the insert. The values plotted are the means of three replicates.

Fig. 36. Light saturation curves for developing mutant chloroplasts from seedlings etiolated for 6 days. The photoreductive activities have been normalized to a value of 1.0 at the maximum light intensity. Data are shown for chloroplasts greened for 2 hr (●), 6 hr (■), 12 hr (▲) and 24 hr (◆) as well as for chloroplasts from seedlings grown for 6 days under continuous illumination (○). The double reciprocal plots of the actual data are shown in the insert. The values plotted are the means of three replicates.

Fig. 37

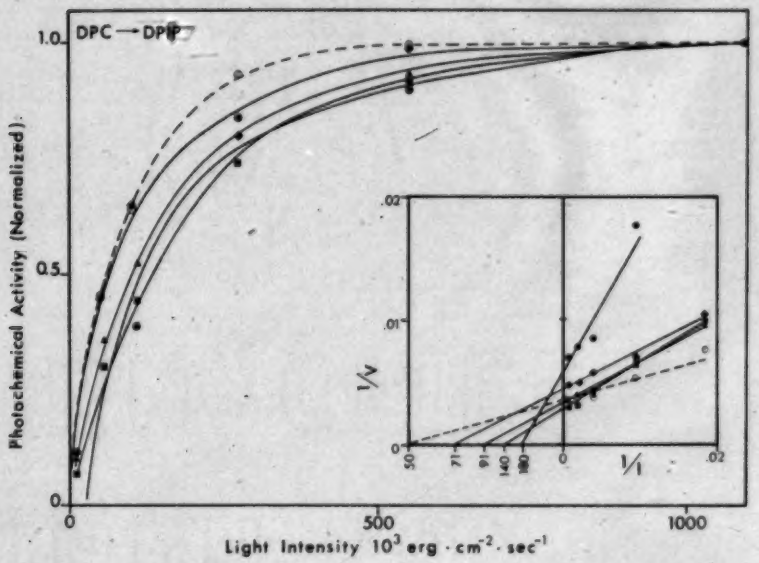

Fig. 38

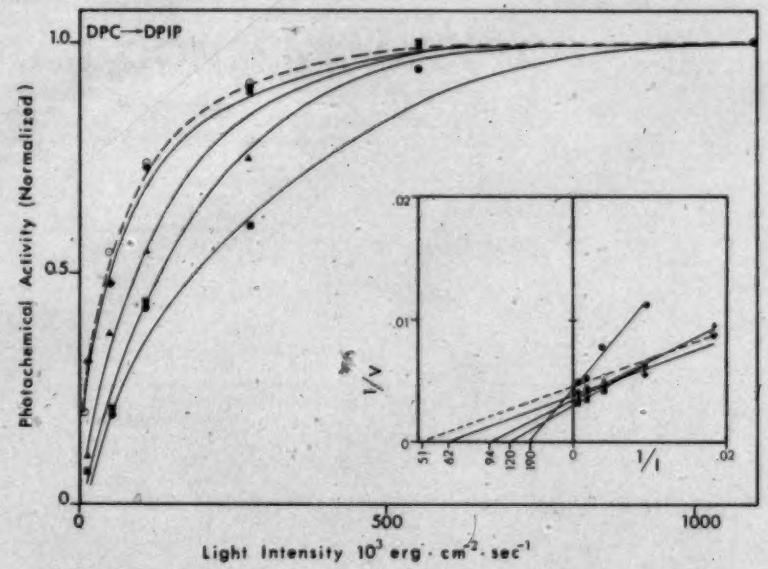

Fig. 37. Light saturation curves for developing normal chloroplasts from seedlings etiolated for 8 days. The photoreductive activities have been normalized to a value of 1.0 at the maximum light intensity. Data are shown for chloroplasts greened for 2 hr (●), 6 hr (■), 12 hr (▲) and 24 hr (◆) as well as for chloroplasts from seedlings grown for 8 days under continuous illumination (○). The double reciprocal plots of the actual data are shown in the insert. The values plotted are the means of three replicates.

Fig. 38. Light saturation curves for developing mutant chloroplasts from seedlings etiolated for 8 days. The photoreductive activities have been normalized to a value of 1.0 at the maximum light intensity. Data are shown for chloroplasts greened for 2 hr (●), 6 hr (■), 12 hr (▲) and 24 hr (◆) as well as for chloroplasts from seedlings grown for 8 days under continuous illumination (○). The double reciprocal plots of the actual data are shown in the insert. The values plotted are the means of three replicates.

from 180 to 71 Kerg/cm²/sec over the first 24 hr of greening in the chloroplasts isolated from N plants etiolated for 8 days. This rate of decrease was closely paralleled by the M (190 to 62 Kerg/cm²/sec) over the same time period. Thus, little difference in the course of development could be seen between the N and M seedlings etiolated for 8 days.

5. DCMU Saturation Measurements

A further characterization of the photosynthetic unit size was undertaken through a study of the kinetics of DCMU inhibition of the photochemical activity of the DPC --> DPIP reaction. The inhibition curves for the N and M chloroplasts from 6 day old seedlings grown under continuous illumination are shown in Figure 39. Although the photochemical activity in the absence of DCMU is higher in the N chloroplasts, the concentration of DCMU required for 50% inhibition of the activity is the same for both N and M plastids. As the inhibition pattern exhibits sigmoidal kinetics, the 50% inhibition point (I50) is more clearly seen in a Hill replot of the data where v=1/2 Vmax = I50 at log (v/(Vmax-v)) =0. Both N and M curves intersect the zero point in this replot at a DCMU concentration of 160 nM. The slope of the Hill equation yields a value for the Hill coefficient which was taken as an estimate of the number of binding sites per molecule, or in this case, per electron transport chain. The slopes of the curves in the Fig. 39 replot are 0.95, suggesting that there was only one binding site for DCMU per electron transport chain.

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Fig. 39

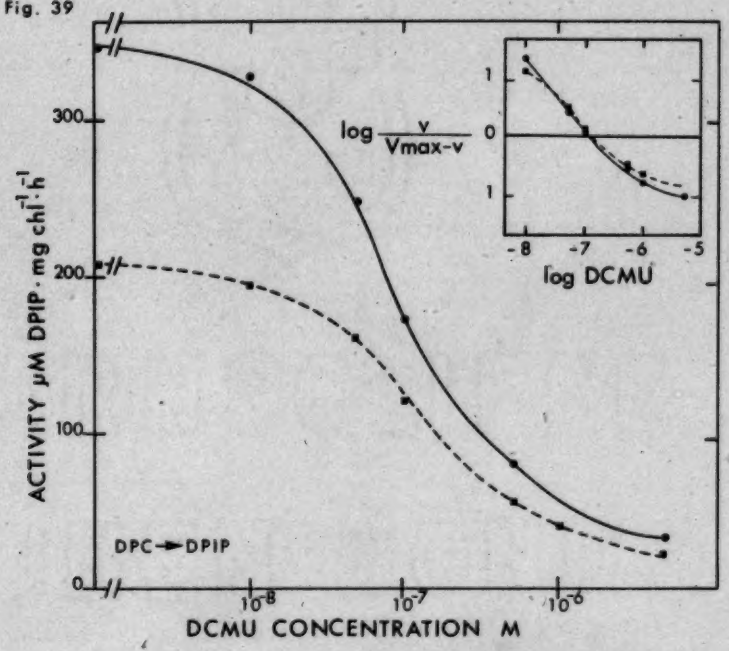

Fig. 40

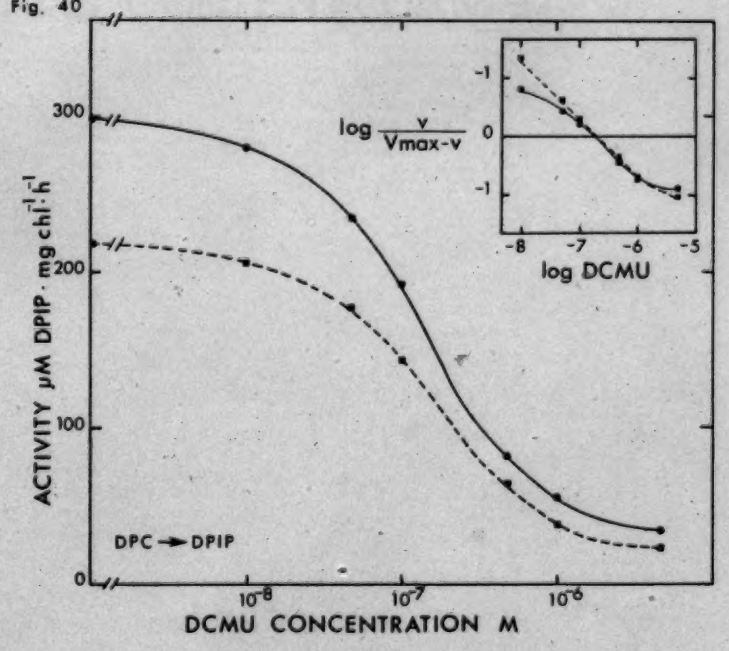

Fig. 39. DCMU inhibition curves for chloroplasts from 6 day normal and mutant seedlings. Photoreductive activities of the DPC-->DPIP reaction are given for normal (●), and mutant (■) chloroplasts. The Hill replot of the data is shown in the insert. The values plotted are the means of four replicates.

Fig. 40. DCMU inhibition curves for chloroplasts from 8 day normal and mutant seedlings. Photoreductive activities of the DPC-->DPIP reaction are given for normal (●), and mutant (■) chloroplasts. The Hill replot of the data is shown in the insert. The values plotted are the means of four replicates.

When plants greened under continuous illumination for 8 days were measured, the data (Fig. 40) exhibited similar patterns to that of the 6 day old plants. After 8 days, the initial activity of the N in the absence of DCMU was lower than it was for 6 days, whereas that of the M had changed very little. Again, the N and M activities reached the 50% inhibition points at the same DCMU concentration. The DCMU I50 after 8 days of continuous illumination was about 200 nM, and the slopes of the Hill plots were 1.0. The increase in concentration of DCMU required for 50% inhibition of activity (on a chlorophyll basis) at 8 days suggested that the photosynthetic units had decreased slightly in size from the 6 day measurement.

The effect of DCMU on chloroplasts from seedlings etiolated for 6 days and then allowed to green is shown in Fig. 41 for N plastids and Fig. 42 for M plastids. The data presented in these figures have been normalized so that the activity in the absence of DCMU is equal to 1.0 and that in the presence of 10 mM DCMU is equal to zero. Lower concentrations of DCMU were required for 50% inhibition of activity in less developed chloroplasts. These values increased from 50 to 100 nM for the N and 8 to 150 nM from the M plastids during the first 24 hr of greening. Since the concentration of DCMU required to inhibit the activity of a unit of chlorophyll increased, the functional photosynthetic unit size must have decreased in these greening plastids. It is also noteworthy that the slope of the Hill plots

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Fig. 41

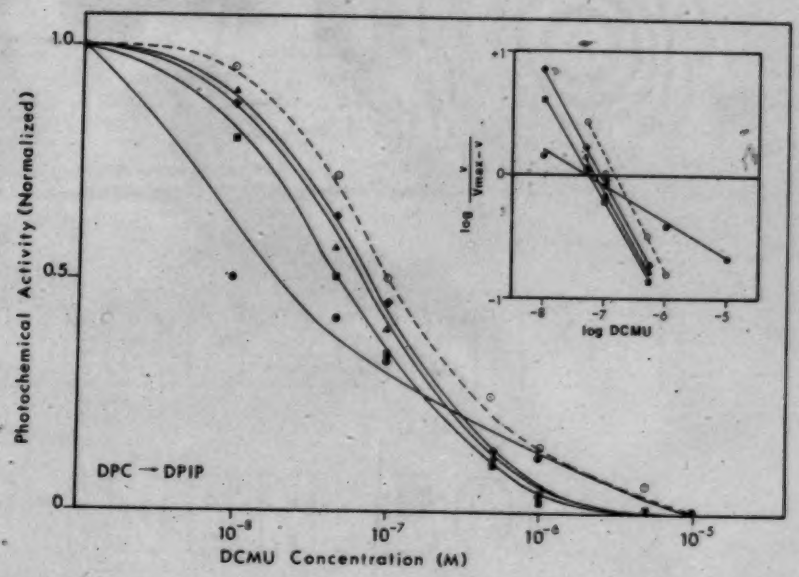

Fig. 42

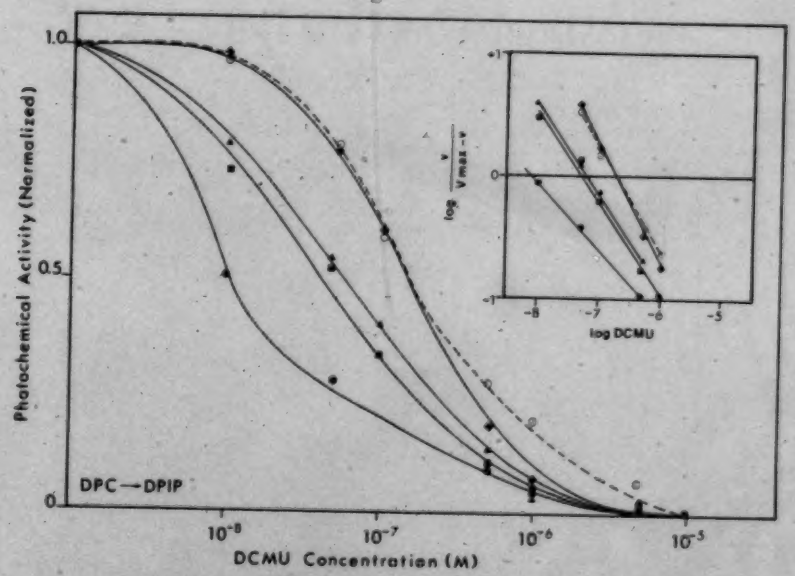

Fig. 41. DCMU inhibition curves for developing normal chloroplasts from seedlings etiolated for 6 days. The photoreductive activities have been normalized to a value of 1.0 in the absence of DCMU and zero activity at 10 mM DCMU. Data are shown for chloroplasts greened for 2 hr (●), 6 hr (■), 12 hr (▲) and 24 hr (◆) as well as for chloroplasts from seedlings grown for 6 days under continuous illumination (○). The Hill plots of the actual data are shown in the insert. The values plotted are the means of three replicates.

Fig. 42. DCMU inhibition curves for developing mutant chloroplasts from seedlings etiolated for 6 days. The photoreductive activities have been normalized to a value of 1.0 in the absence of DCMU and zero activity at 10 mM DCMU. Data are shown for chloroplasts greened for 2 hr (●), 6 hr (■), 12 hr (▲) and 24 hr (◆) as well as for chloroplasts from seedlings grown for 6 days under continuous illumination (○). The Hill plots of the actual data are shown in the insert. The values plotted are the means of three replicates.

approximate unity for all but the 2 hr N sample, in which case it was 0.59.

The results from seedlings etiolated for 8 days and then allowed to green are shown in Figures 43 and 44 for N and M chloroplasts respectively. These results are similar to those for 6 day etiolated seedlings in that the DCMU I50s increased as greening progressed. In N plastids, these values increased from 14 to 200 nM over the first 24 hr of greening, and from 40 to 200 nM in the M (2 hr M values could not be determined). The Hill coefficient for all curves approximated unity except for the 2 hr N sample which was 0.63. The consistently low Hill coefficients in very young samples may suggest a substantial amount of DCMU insensitive photoreduction of DPIP.

C. Discussion

Studies on the development of photochemical activities during greening are confounded by the changing levels of chlorophyll on which they are usually based. Since the chlorophyll base value changed (Fig. 1), its usefulness as a developmental parameter was questioned. The protein content of the plastids during development was more stable (Figures 4 and 5) and was, therefore, considered to be a more useful base for the expression of development of photochemical activities. However, the activity expressed on a unit chlorophyll was useful in detecting the onset of photochemical activity. In addition, the decline in activity

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Fig. 43

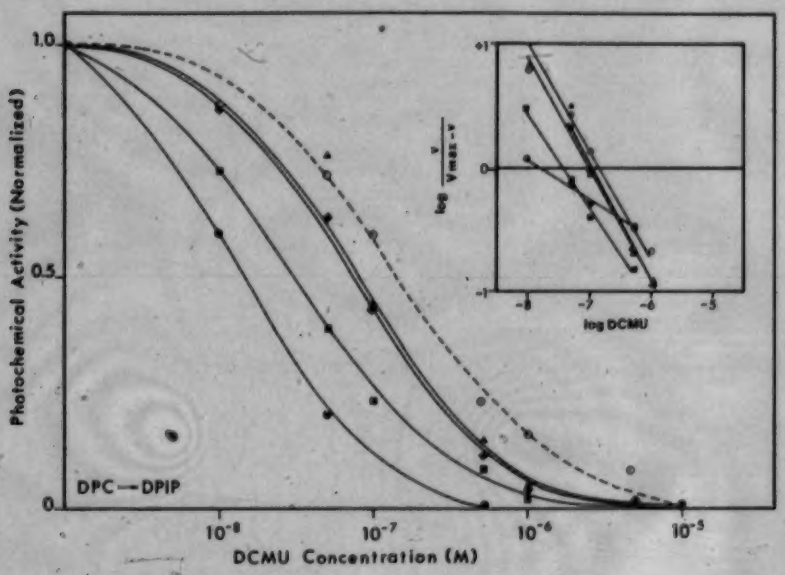

Fig. 44

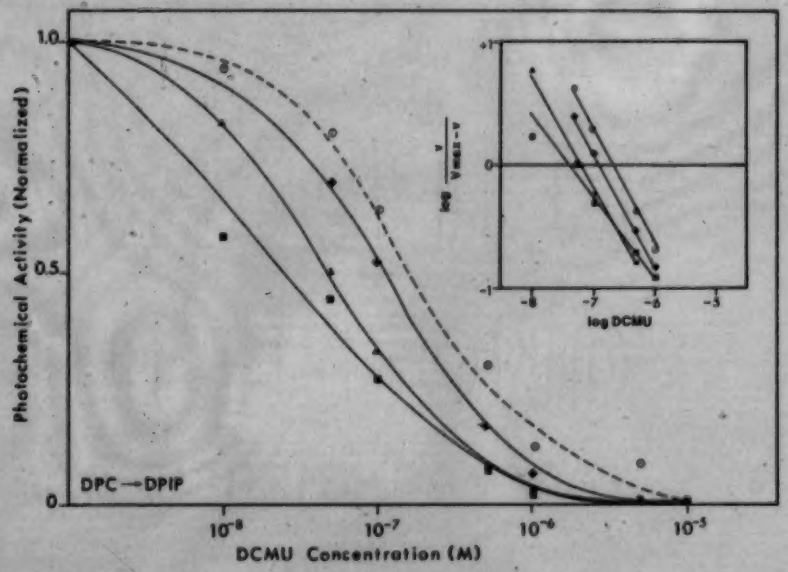

Fig. 43. DCMU inhibition curves for developing normal chloroplasts from seedlings etiolated for 8 days. The photoreductive activities have been normalized to a value of 1.0 in the absence of DCMU and zero activity at 10 mM DCMU. Data are shown for chloroplasts greened for 2 hr (●), 6 hr (■), 12 hr (▲) and 24 hr (◆) as well as for chloroplasts from seedlings grown for 8 days under continuous illumination (○). The Hill plots of the actual data are shown in the insert. The values plotted are the means of three replicates.

Fig. 44. DCMU inhibition curves for developing mutant chloroplasts from seedlings etiolated for 8 days. The photoreductive activities have been normalized to a value of 1.0 in the absence of DCMU and zero activity at 10 mM DCMU. Data are shown for chloroplasts greened for 6 hr (■), 12 hr (▲) and 24 hr (◆) as well as for chloroplasts from seedlings grown for 8 days under continuous illumination (○). The Hill plots of the actual data are shown in the insert. The values plotted are the means of three replicates.

after the sharp rise seen in many experiments (Figures 23 and 29) could be attributed to changes in the photosynthetic unit size (numbers of chlorophylls/reaction centre).

The development of whole photosystem PET was measured by the H2O --> FeCN (O2 evolution) and H2O --> DPIP (DPIP reduction) reaction sequences. The water splitting activity measured by O2 evolution was virtually nonexistent on a protein basis during the first 6 hr of greening in N plastids (Fig. 21 and 22). The plastid chlorophyll content at this time was less than 10% of the fully greened tissue (Fig. 4), and the increase in activity per unit chlorophyll from this point on occurred at a faster rate than that on a protein basis. Activity in the M was slower to develop than in the N and did not appear to be affected by the extended dark period. The development of the water splitting capacity of the N samples was not different between 6 and 8 days of etiolation

The H2O --> DPIP reaction should be a measure of the same chain sequence as H2O --> FeCN, however the results were substantially different. On a protein basis, these activities were measurable within 4 hr of greening (Fig. 25 and 26). As a result of this early onset, the activity per unit chlorophyll showed a characteristic peak and decline to stable levels as chlorophyll accumulated (Fig. 23 and 24). The earlier onset of the H2O --> DPIP reactions may indicate that some compound in the plastid or buffer other than water was responsible for electron donation in these early stages

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of chloroplast development, or may simply reflect the differential accessibility of the acceptors to the membranes during development. Unlike the water splitting measurements, a significant effect was seen in the delay of the greening process by prolonged etiolation (Fig. 23 and 24). After 6 days of etiolation, the activity peaked at 3 and 4 hr for N and M respectively, whereas after 8 days, the activity did not peak until 6 and 9 hr. In addition, the activity stabilized in the first 12 hr of greening in 6 day seedlings, but it took about 24 hr in the 8 day seedlings. On a unit protein basis, the M is unaffected by the prolonged period in the dark, whereas the developmental rate was reduced in the N seedlings (Fig. 26 and 26). In the studies on the accumulation of chlorophyll and the sequential appearance of membrane proteins, it was also found that prolonged etiolation had little effect on the M, whereas it reduced the rate of development of the N.

DPC is an artificial donor to PSII which can function long before the development of the water splitting capacity. High activities of DPIP photoreduction were seen with DPC as electron donor within the first hour of greening (Fig. 27). Thus, even though the water splitting capacity had not yet developed, the PSII traps were present and functional. The initial activities were high because of the small amount of chlorophyll in the system. The rate of decline in the ability to stimulate DPIP photoreduction by DPC occurred faster in the 6 day than in the 8 day etiolated seedlings.

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This would certainly be an expected consequence of an overall reduction in rate of plastid development seen in the N.

Significant PSI activities were observed within the first 2 hr of greening in both N and M seedlings. On a chlorophyll basis, maximum rates of the TMPD --> MV reactions were seen at 8 hr for both N and M after 6 days of etiolation (Fig. 29). After 8 days of etiolation, these maximum rates were achieved in only 4 hr of greening (Fig. 30). The earlier peaking of activity after 8 days of etiolation at first seems to suggest a more rapid development of PSI after prolonged darkness, contrary to the results obtained thus far. It could also, however, be explained by an identical rate of PSI development at both 6 and 8 days, superimposed on the reduced rate of chlorophyll development seen after 8 days of etiolation. The net result would be an earlier peak in activity per unit chlorophyll. This suggestion is supported by the similarity of the curves for PSI development when expressed on a protein basis (Fig. 31 and 32). In addition, the stabilization of the PSI activity on a chlorophyll basis after only 24 hr in the 6 day plants, but after 36 hr in the 8 day plants, indicated that the rate of plastid development was reduced in the 8 day etiolated seedlings.

The PET specifically through PSII was measured by the DPC --> DMQ reaction. This reaction was not coupled, and was insensitive to DBMIB as seen in Table II. The H2O --> DMQ

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sequence is known to be coupled, and P/2e ratios of 0.3 to 0.4 have been reported (82, 230). Following 6 days of continuous illumination, the activity of the M was about 75% that of the N, but after 8 days the activities were about equal if not somewhat higher in the M. The stimulation of activity by the addition of the uncoupler in the absence of DBMIB occurred to the same extent in both N and M chloroplasts. DBMIB prevents electron transport beyond plastoquinone, and DMQ picks up electrons specifically at plastoquinone. A lack of electron acceptors and substrates for photophosphorylation caused an effective blockage beyond plastoquinone, and thus, the activity measurements in the absence of uncoupler did not differ in the presence or absence of the DBMIB.The lack of stimulation by the uncoupler on the DBMIB inhibited PET from DPC to DMQ is explained simply by the fact that no coupling site is contained within the sequence. The stimulation of the system by the uncoupler in the absence of DBMIB could be explained by one of two possibilities. If reduced plastoquinone can transfer electrons to benzoquinone, then a functional cyclic PET through PSI which is coupled, might contribute to the additional reduction of DMQ. The second possibility is that in the absence of DBMIB, oxygen may act as an electron acceptor for PSI in a pseudocyclic electron transport. Pseudocyclic PET results in oxygen uptake by a Mehler reaction which would be indistinguishable from the reaction of reduced DMQ. In both cases, the coupled activity in the

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absence of DBMIB must be slow enough to cause a backlog of reduced PET chain intermediates resulting in a diversion of most of the electrons to DMQ.

The lower chlorophyll concentrations seen in the M may be due to less chlorophyll associated with each reaction centre (smaller photosynthetic unit sizes), or to fewer reaction centres (no change in the photosynthetic unit size) per unit of leaf weight. To determine whether or not the photosynthetic unit sizes varied between N and M, the kinetics of light saturation of the DPC --> DPIP reaction were studied. The convergence of the reciprocal plots on the ordinate in figures 33 and 34 indicate that the photosynthetic unit sizes of the N and M were the same at both 6 and 8 days. If the slope of the linear portion of the light-limiting region of the light saturation curves directly defines the efficiency or quantum yield of the reaction (97), then clearly the M had a lower quantum yield at the 6 and 8 day stages, although it recovered somewhat at the 8 day stage.

Decreases in the light intensity required for saturation fo the DPIP photoreduction occurred concomitantly with chloroplast development. Similar changes have been reported in Chlorella for photosynthetic oxygen evolution as a function of greening (97, 242). Half-saturation intensities of 300 Kerg/cm²/sec have been reported for the H2O --> TPIP reactions in seedlings of barley greened for 2 hr, and 50 Kerg/cm²/sec after 24 hr of greening (98). A

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decrease in light energy required for saturation of photosynthesis suggests an increase in size of the photosynthetic unit during greening (17). After 6 days of etiolation, the light intensity required for saturation of the developing M plastids decreased more slowly than in the N. After 8 days however, these rates were almost the same for the N and M due mainly to the reduced rates of development of the N.

The DCMU saturation kinetics were also studied to compare the photosynthetic unit sizes. In light-grown seedlings, no difference was seen between N and M plastids in the amount of DCMU required to inhibit the activity of a unit of chlorophyll by 50%. These values were approximately 160 nM and 200 nM for 6 and 8 days respectively. In the 3.0 ml reaction cuvette the absolute amount of DCMU for the I50 would be 0.48 and 0.60 nMol. If one molecule of DCMU inhibits 1 reaction centre, then the number of reaction centres in a mg of chlorophyll should be 2 x I50 or .98 and 1.20 nMol per mg chlorophyll. In other words 1 DCMU per 920 and 750 chlorophylls for 6 and 8 day old seedlings. The assumption that 1 molecule of DCMU binds per reaction centre is born out by the Hill plots which have a slope of one. The values for the number of inhibitor sites agree with those of similar experiments reported by Izawa and Good (112). They also pointed out, however, that a small portion of the DCMU is irreversibly bound to the chloroplast membrane with no inhibition of photochemical activity.

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The number of chlorophylls per inhibitor site (reaction centre) decreases for both N and M fully green tissue between 6 and 8 days. After 6 days of etiolation and 2 hr of greening, the values were 1 DCMU inhibition site per 3000 (N) and 18000 (M) chlorophylls. These values may indeed indicate a decrease in the photosynthetic unit size during greening contrary to the results from the light saturation curves, however, they may more probably reflect a change in sensitivity to DCMU during greening. A change in DCMU sensitivity as a function of greening has been reported previously in barley (98). Substantial amounts of DCMU-insensitive photochemical activity were seen in all the photochemical measurements performed on very young chloroplasts, and care was taken to compensate for the DCMU-insensitive component. The change in DCMU sensitivity was also suggested by the decrease in the Hill slopes to values less than 1.0 in the early stages of greening (Fig. 41 and 43).

The I50 concentrations in greening seedlings after 6 days of etiolation were usually lower in the M than in the N chloroplasts and approached the light grown control values more slowly. After 8 days of etiolation, the approach to the light grown values occurred at about the same rates for the N and M due to a marked reduction in the rate of development of N. These generalities seem to concur with the basic finding that the rate of plastid development, although slower in the M after 6 days of etiolation, was almost the

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same as the N after 8 days of development due to a reduction in the rate of N chloroplast development at the 8 day stage.

Both the DCMU inhibition and the light saturation curve data indicated that the photosynthetic unit size was the same for N and M seedlings after 6 and 8 days of development in continuous light, and that the lower photochemical activities of the M were due to a decreased efficiency or quantum yield.

Freeze-fracture experiments indicated thatthe overall EF and PF particle sizes were smaller in the M (see appendix). Thus, the size of the freeze-fracture particles did not appear to have a direct relationship to the size of the photosynthetic unit. However, the reduction in size of the M EF and PF particles may have affected their orientation with each other, to the extent that the efficiency of the system was reduced.

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V. APPENDIX:FREEZE-FRACTURE STUDIES

A. Materials and Methods

Chloroplasts were prepared from normal and mutant barley seedlings grown for 6 or 8 days under continuous illumination as described previously in this thesis. Glycerol was slowly added to the isolated chloroplast membrane preparations over a period of 30 min at 5C to give a final concentration of 33% v/v. The samples were centrifuged at 8000 g for 4 min to yield a pellet with a paste-like consistency. Portions of the pellet were frozen in liquid Freon 22 and processed in a Balzers BA 360 M high vacuum freeze etch unit. Fracturing was carried out at -100C and the fractured surface was unidirectionally shadowed with Pt followed by C evaporation. The freeze fracture replicas were examined in a Phillips EM 300.

Thin sections were also prepared from the glycerol-impregnated chloroplast pellets used for freeze-fracture electronmicroscopy. The glycerol-impregnated pellets were compacted by centrifugation at 50,000 g for 10 min. Plugs of the pellet were removed with a Pasteur pipette and fixed in 2% glutaraldehyde in resuspension buffer (50 mM HEPES (pH 7.6); 0.33 M sorbitol; 2 mM EDTA; 1 mM $MgCl_2$; and 1 mM $MnCl_2$) at 0C for 1 hr. All subsequent steps were carried out as described for the leaf tissues.

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Particle sizes and distributions were determined as described by Staehelin (214). All electron micrographs were taken at the same magnification step on the microscope and photographically enlarged to give a total magnification of 74,000. The particle diameters were measured under a 10 x binocular microscope fitted with an occular containing a micrometer scale. From 40 to 60 separate fracture faces (2-5 um^2 of membrane surface) were analyzed from thylakoids isolated from 6 and 8 day old N and M seedlings grown under continuous illumination. Measurements of not less than 700 particles were made for the size frequency histograms. The particle densities (number of particles per um^2 of membrane surface) were determined by counting the number of particles on a particular fracture face and dividing this by the area of the face determined from the weight of a piece of transparent gravimetric paper, which was traced and cut out in the shape of the fracture face.

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B. Results

The standard chloroplast isolation procedure resulted in the preparation of destacked thylakoid membranes as shown by the EM thin section of a chloroplast pellet (Fig. 45). As a result, the freeze-fracture replicas showed no identifiable stacked regions for either N (Fig. 46) or M (Fig. 47) chloroplasts. The EF particle size ditributions for N and M plastids after 6 and 8 days of development are shown in Fig. 48. Thylakoids of the N seedlings had a

Fig. 45

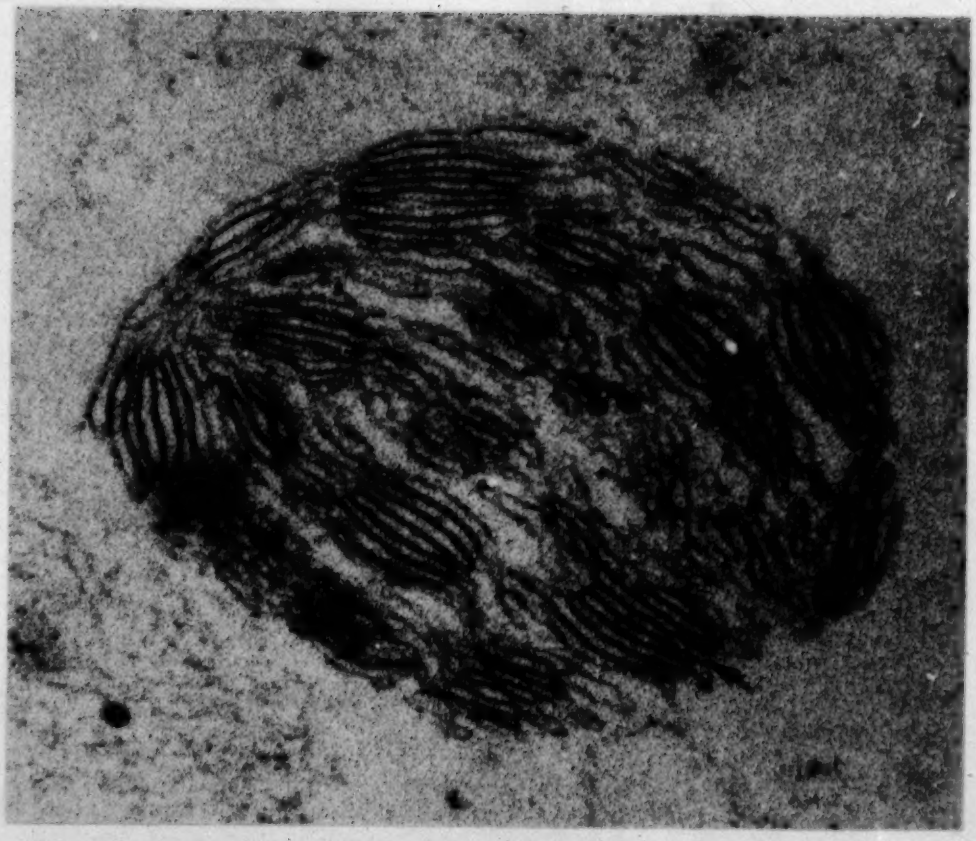

Fig. 45. TEM thin section of chloroplast pellet used for the freeze-fracture analysis. Plastid preparations lacked a chloroplast envelope and were clearly destacked as indicated by the arrows. Magnification, X35,000.

Fig. 46

Fig. 47

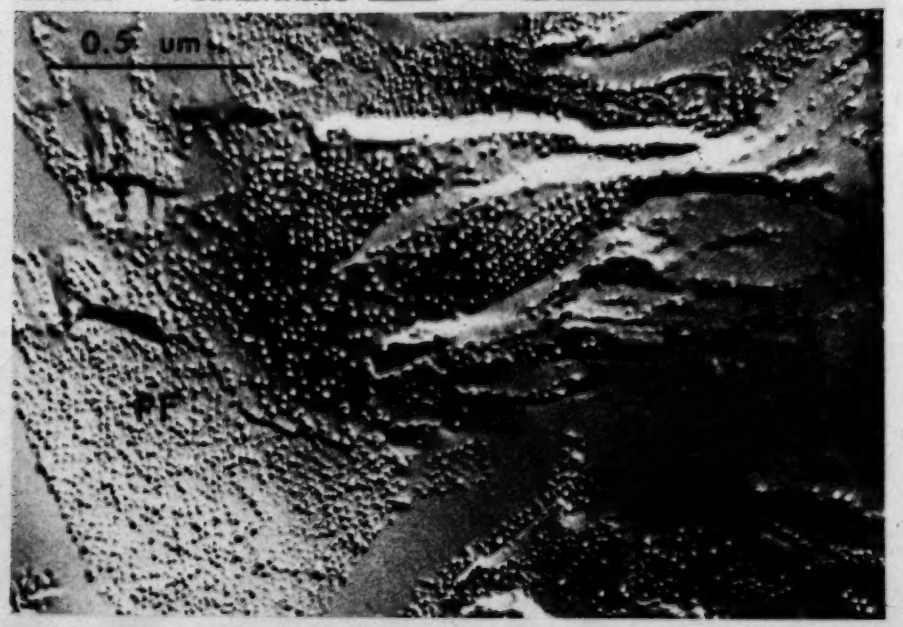

Fig. 46. Freeze-fracture faces of thylakoids from normal barley seedlings grown for 6 days under continuous light. EF, endoplasmic face; PF, protoplasmic face.

Fig. 47. Freeze-fracture faces of thylakoids from mutant barley seedlings grown for 6 days under continuous light. EF, endoplasmic face; PF, protoplasmic face.

bimodal size distribution with modal means of about 140A and 175A after both 6 and 8 days of continuous illumination. The thylakoid membranes of the M however, were totally devoid of the 175A particle size class and no change was apparent between 6 and 8 day old seedlings.

The PF particles of destacked N and M chloroplasts also fell into 2 size classes (Fig. 49). These classes had modal means of about 106A and 134A. In contrast to the EF particles, the PF particle size distributions changed significantly between 6 and 8 days. Thylakoid membranes of the N seedlings exhibited a bimodal pattern at the 6 day stage whereas the PF particles from M membranes were totally in the smaller size class. After 8 days of continuous illumination most the PF particles of the N were in the larger size class and the appearance of the larger size class in the M resulted in a bimodal pattern similar to that seen for the N after 6 days. In both cases it appeared that an increase in the size of the PF particles was related to chloroplast development.

The freeze-fracture particle densities (particles per um^2) are presented in Table III. No significant difference was seen in the particle density of the EF between membranes isolated from N and M. In addition, there was no significant change in density of the EF particles between 6 and 8 days of continuous illumination. A considerably higher density of PF particles in the M relative to the N was obtained at the 6 day stage, but by 8 days the packing densities were

Fig. 48

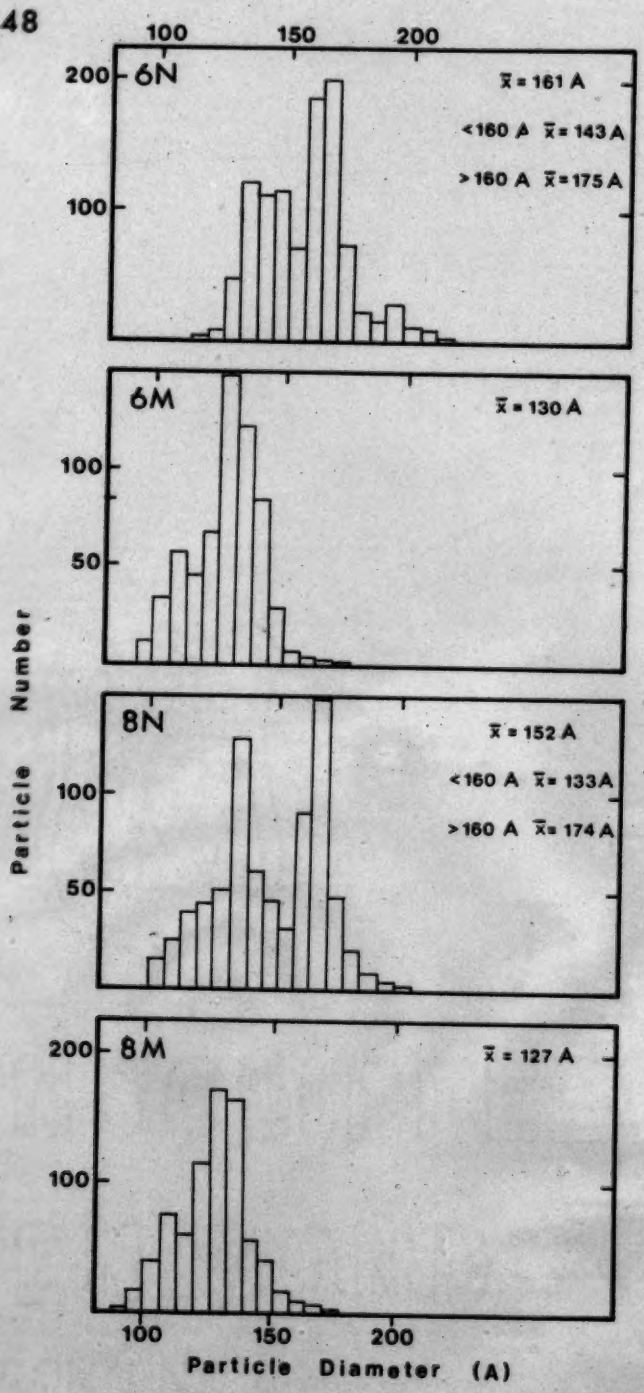

Fig. 48. EF particle size distributions on thylakoids from 6 and 8 day normal and mutant seedlings. Overall mean particle diameters as well as the modal means are presented.

Fig. 49

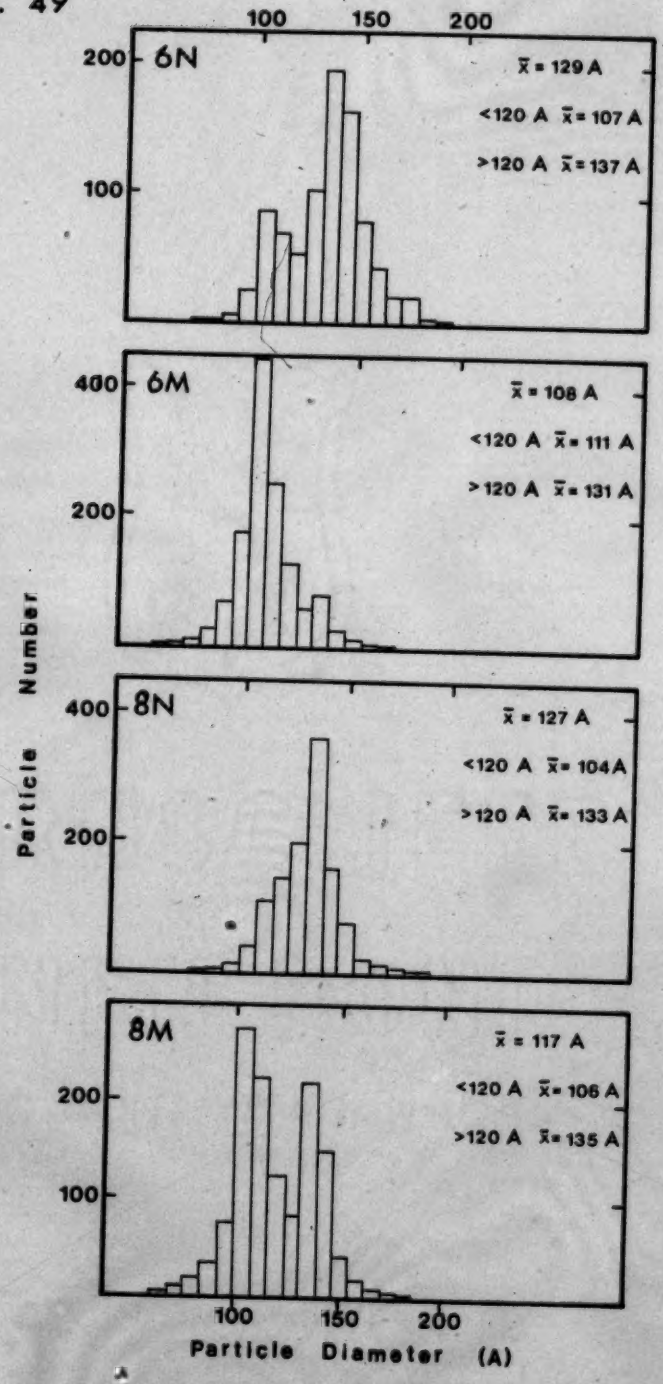

Fig. 49. PF particle size distributions on thylakoids from 6 and 8 day normal and mutant seedlings. Overall mean particle diameters as well as the modal means are presented.

Table III. Freeze-fracture particle densities on chloroplast membranes from N and M seedlings grown for 6 or 8 days in continuous light.

Sample	Faces Counted	Total area um^2	Particle Density particles/um^2 ± S.E.
6 day N EF	45	4.98	554 ± 20
6 day M EF	43	4.53	560 17
8 day N EF	41	1.94	649 24
8 day M EF	43	5.27	615 24
6 day N PF	37	3.02	1655 38
6 day M PF	57	3.38	2118 35
8 day N PF	46	1.74	3249 75
8 day M PF	57	2.43	3030 93

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slightly higher in the N. Between 6 and 8 days the PF particle packing density of the N increased 50% whereas an increase of only 30% occurred in the M over the same time period. The higher proportion of smaller size particles in the M at 6 days may be responsible for the higher packing density seen in Table III.

C. Discussion

More than one EF particle size class has been previously reported in peas (10), spinach (214), Euglena (81) and Chlamydomonas (175), but only one size class has been reported for barley (159, 211). The two size classes found were somewhat larger than those reported in other species which exhibit bimodal patterns. The M EF particle size distribution, however, was similar to that previously described for a chlorophyll b-deficient M of barley (159). The PF particles of chloroplasts from 6 day N seedlings had a bimodal distribution, but in the more mature 8 day tissue, only the larger class was seen (Fig. 49). Thus, it was apparent that the PF particle size distribution is affected by the developmental age of the tissue making comparisons of data from different sources difficult. The M, which showed a bimodal pattern after 8 days had only the smaller size class after 6 days of greening. Thus, it appears that a developmental sequence in transition of small (106A) PF particles to large (134A) PF particles was occurring and

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that this transition was lagging in the M. In addition, the particles of the M membranes did not fall into different modal size classes, but rather, the difference between the N and M membranes was the relative proportion of particles in each of those classes.

The larger size class of EF particles (175A) was completely absent from M membranes (Fig. 48) at both 6 and 8 day stages. The bimodal pattern of the N membranes did not appear to change over the 2 day period as it did for the PF particle distributions. A LHC-deficient M of barley was shown to exhibit only a small size class of EF-particles (159), but in the case of the virescens M, abundant amounts of LHC were present (Fig. 15). Thus, although the lack of LHC might cause a decrease in size of the EF particles, a decrease in size of EF particles is not necessarily due to a lack of LHC.

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